U.S. Fish & Wildlife Service

California-Nevada Fish Health Center Investigational Report:

Myxosporean Parasite (*Ceratonova shasta*) Prevalence of Infection in Klamath River Basin Juvenile Chinook Salmon, March – August 2023

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Summary

Juvenile Klamath River Chinook Salmon (*Oncorhynchus tshawytscha*) were assayed for myxosporean parasite *Ceratonova shasta* from late March through mid-August 2023 by quantitative polymerase chain reaction (QPCR). Histology testing was not conducted in 2023.

Annual *C. shasta* prevalence of infection (POI) increased in 2023, compared to 2022. The annual *C. shasta* POI in 2023 by QPCR was 42%, compared to 39% in 2022.

Natural fish were monitored in real-time for the first 11 weeks of the season to provide timely data to water resource managers and basin cooperators. *Ceratonova shasta* was first detected by QPCR in fish sampled the week of April 30 (37% POI) from the Kinsman rotary screw trap (~ river mile 147).

In late May, natural fish collected from Kinsman had high *C. shasta* POI (93-95%), and 27% of those fish had severe infections based on the DNA copy number (> 3 logs of *C. shasta* DNA. The majority of fish collected in 2023 were of natural origin.

During real-time monitoring, gill tissue was also collected and tested at a later date. *Ceratonova shasta* detection in gill occurred at the same time as infection in the intestine. Gill tissue, in conjunction with Oregon State University water sampling data, may be a more informative real-time metric to assess disease risk in the Klamath River.

Iron Gate Hatchery released Chinook Salmon smolts into the Klamath River starting on June 8, 2023. Coded-wire tagged (CWT) Chinook Salmon originating from Iron Gate Hatchery were collected in the Klamath River from June 13 to August 16. The highest *C. shasta* prevalence of infection occurred in fish 3 weeks at large (WAL), while the mean DNA copy number was highest at 2 WAL.

In 2023, *C. shasta* was undetected in March and April, however infection did occur later in the year, especially during June and July. Infection occurred later and at a higher magnitude, compared to 2022. This could be related to cooler spring water temperatures, flow, or exposure dose which are all connected factors that influence infection and severity in salmonid.

The correct citation for this report is:

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Notice

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Introduction

Historically Klamath River juvenile Chinook Salmon have experienced high prevalence and severity of infection with two myxosporean parasites, *Ceratonova shasta* (*syn. Ceratomyxa shasta*, Atkinson et al., 2014) and *Parvicapsula minibicornis*. *Ceratonova shasta* causes enteronecrosis and contributes to the mortality of juvenile salmonids that migrate through the region (Bartholomew et al., 1997; Stocking et al., 2006).

The life cycle of *Ceratonova shasta* includes a freshwater annelid host, *Manayunkia occidentalis* (Atkinson et al., 2020), as well as the vertebrate salmon host. Infected annelids release actinospores into the water where they attach to the salmon's gill epithelium, invade the blood, and migrate to the intestinal tract for further multiplication and sporogony. The actinospore develops within the vertebrate host, salmon or trout species, into a myxospore (Hallett et al., 2012). Once shed from an infected fish, the myxospore can infect the annelid host to complete the life cycle (Bartholomew et al., 1997). Infected fish can develop varying degrees of enteritis depending on actinospore density, host resistance, and water temperature (Udey et al. 1975; Foott et al. 2004; Bjork and Bartholomew 2009; Ray et al. 2010; Hallett et al. 2012; Foott et al. 2023).

The California-Nevada Fish Health Center has performed standardized monitoring of *C. shasta* POI in Klamath River juvenile Chinook Salmon since 2009. The primary objectives of monitoring in 2023 were to 1) examine *C. shasta* POI and infection severity (parasite DNA quantity within fish tissue) in Klamath River juvenile Chinook Salmon during the spring out-migration period, and 2) compare parasite prevalence to previous years (2009-2022).

Methods

Pre-Release Examination

Prior to the first Iron Gate Hatchery (IGH) release on June 8, a fish health examination of 30 fall-run Chinook Salmon was conducted (May 18, 2023) to determine infection levels of *C. shasta.*

Collection Reaches

The lower Klamath River basin is divided into six reaches that are defined by major tributaries, with study cooperators collecting fish in each reach (Table 1).

Table 1. Reach locations, rotary screw trap locations, distances, and cooperating agencies performing fish collection on the main-stem Klamath River.

Collection Reach Klamath River main stem	Reach Code	River miles (Upstream – Downstream)	Collector
Iron Gate Dam to Shasta R.	K5	190-177	USFWS
I-5 rotary screw trap[1]		~183	USFWS
Shasta R. to Scott R.	K4	177-144	USFWS
Kinsman rotary screw trap[1]		~ 147	USFWS/Karuk Tribe
Scott R. to Salmon R.	K3	144-66	Karuk Tribe
Salmon R. to Trinity R.	K2	66-44	Karuk Tribe
Weitchpec rotary screw trap[1]		~ 44.2	USFWS/Yurok Tribe
Trinity R. to Blue Creek	K1	44-16	Yurok Tribe
Blue Creek to Estuary	K0	16-0	Yurok Tribe

[1] Primary collection site within that reach

Typically, sample collection begins in late March and concludes in late August as fish migrate out of the basin and the weekly sample size target can no longer be met. In 2023, fish health sampling concluded the week of August 13.

In 2023, fish were collected in the main-stem Klamath River between the I-5 RST and Klamath Glen in the K1 reach (a distance of ~176 river miles). Rotary screw traps were used for fish collections, when possible. However, beach seining was also performed to collect fish in some weeks/reaches.

Fish were collected the week of March 19 – June 18 in the upper reaches (K5 and K4) and collected the weeks of April 2 – July 30 from reaches K3 and K2. Fish were also collected below the Trinity River confluence (reach K1 and K0) later in the sampling season (weeks of June 25 – August 13).

Fish Origin

All fish collected were categorized into groups based on their origin. A Chinook Salmon of natural origin is unmarked (adipose fin present) and collected prior to hatchery release. Unmarked salmon collected after hatchery release are classified as unknown origin.

Fish collected in the Klamath River varied by reach, with an emphasis on natural fish in the reaches below Iron Gate Dam before hatchery release, then available hatchery CWT fish for the remainder of the spring/summer migration.

In 2023, the majority of fish collected for fish health testing were of natural origin. Fish collected after June 8 were from the combined natural and hatchery populations and the ratio of the two populations in the fish health samples was unknown.

Gill Tissue Vs Intestinal Tissue by QPCR

Ceratonova shasta results from QPCR testing of intestinal tissue were reported in real-time for fish collected from the Kinsman RST in the Shasta River to Scott River reach (K4). In 2023, gill tissue was also collected from this subset of fish and archived for testing at a later date. All gill arches from an individual fish were excised, DNA extracted, and assayed using the same methodology as intestinal tissue (Voss et al. 2020).

Data Analysis, Necropsy, DNA Extraction, and Assays

Prevalence of infection (POI) is used to describe the proportion of infected Chinook salmon (numerator) in the sample (number of animals examined). Refer to Appendix A for definitions and data analysis.

Fish necropsy and DNA extraction were modified from Voss et al., 2020 to exclude the excision of kidney tissue. Kidney tissue was not tested by QPCR in 2022 or 2023. *Ceratonova shasta* was only assayed by quantitative PCR in 2023. Refer to Appendix B for detailed methodology on quantitative PCR.

In 2023, all QPCR assays were conducted using an ABI QuantStudio 6 Flex Real-Time PCR instrument. This instrument model was upgraded last year. For more information on the transition of the *C. shasta* QPCR assay to this upgraded instrument model, refer to Voss et al., 2023.

Results and Discussion

Pre-Release Examination of Iron Gate Hatchery Chinook Salmon

Ceratonova shasta was not detected in 30 juvenile Chinook Salmon collected from Iron Gate Hatchery on May 18, 2023.

Number of Fish Collected

In 2023, we tested 1,790 juvenile Chinook Salmon collected from the main-stem Klamath River. Natural fish accounted for 63% (n =1132), fish of unknown origin accounted for 13% (n = 234), and hatchery fish accounted for 24% (n = 424) of fish collected (Figure 1). The number of fish collected weekly is presented in Appendix C.

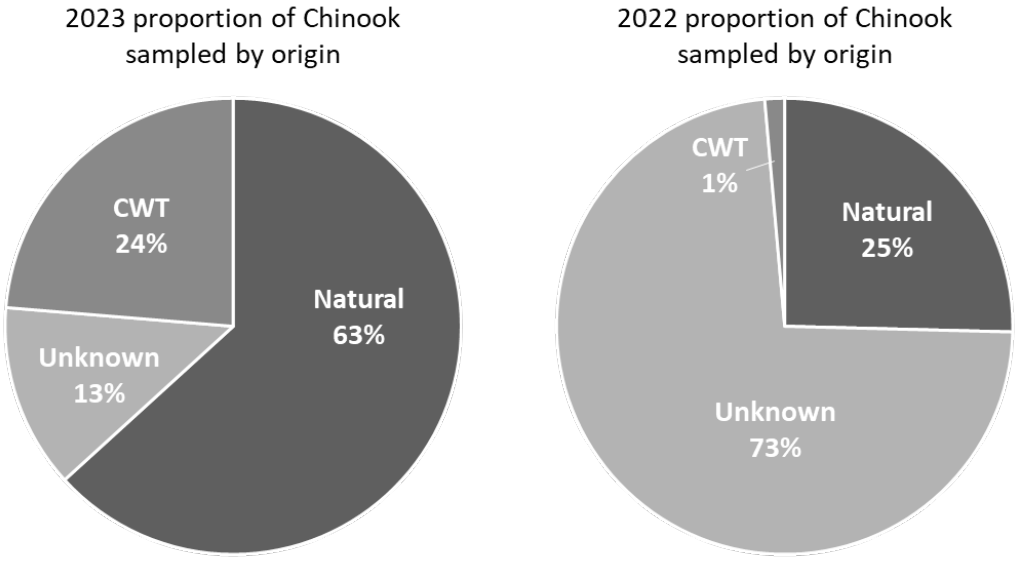

Figure 1. Comparison of the proportion and origin of Chinook salmon used for prevalence of infection analysis. The pie graph on the left is the proportion of fish sampled (N=1790) in 2023. The pie graph on the right is the proportion of fish sampled (N =1571) in 2022. Unreadable tag codes or lost tags, and TRH tags have been removed from the total number of fish collected.

The proportion of natural and hatchery fish increased in 2023, compared to 2022. In 2023, Iron Gate Hatchery released fish in June which allowed time in the spring for natural fish to be collected for fish health testing. Last year, the hatchery release occurred in April and therefore the amount of time to collect natural fish was shortened in 2022. There were also fewer fish of unknown origin in 2023 due to IGH release timing.

Annual Prevalence of Infection

The annual prevalence of *C. shasta* infection in all juvenile Chinook Salmon intestinal tissue tested in 2023 by QPCR was 42% (756/1,790, confidence interval [ci] = 40-45%). *Ceratonova shasta* was first detected by QPCR in fish sampled the week of April 30 in the Shasta River to Scott River reach (K4, Appendix C, Table C-1). Overall, the annual *C. shasta* POI by QPCR was similar in 2023, compared to 2022 (39%, ci = 37-42%).

Annual *C. shasta* POI was highest in the Salmon River to Trinity River (K2) reach at 88%, followed by 78% in the Trinity River to Blue Creek (K1) reach. The lowest annual *C. shasta* POI in 2023 was in the Iron Gate Dam to Shasta River (K5) reach at18% (Figure 2).

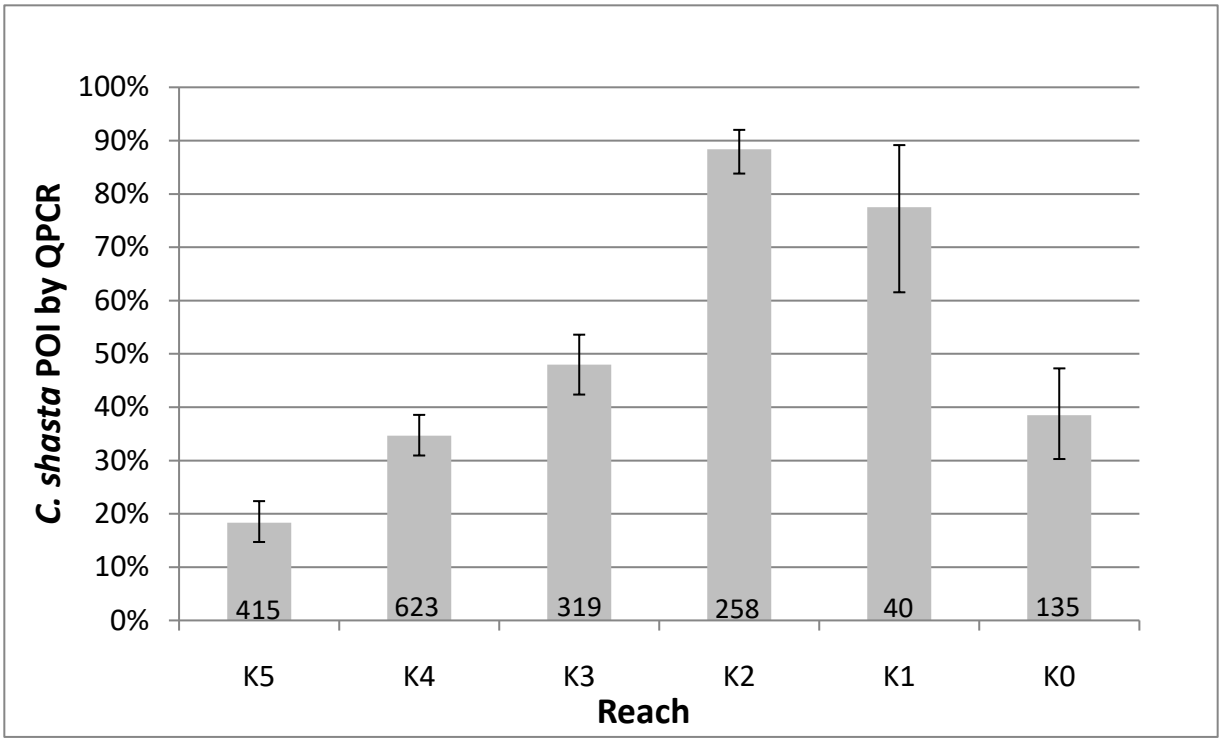

Figure 2. Prevalence of *Ceratonova shasta* infection by reach in juvenile Klamath River Chinook Salmon tested by QPCR in 2023. Iron Gate Dam to Shasta River (K5), Shasta River to Scott River (K4), Scott River to Salmon River (K3), Salmon River to Trinity River confluence (K2), Trinity River to Blue Creek (K1), and Blue Creek to Klamath River Estuary (K0). Sample size collected in each reach are displayed inside the bar graph. Error bars represent a 95% confidence interval.

Monitoring of waterborne stages of *C. shasta* from river water conducted by Oregon State University (OSU) showed a pattern of low spore density in the spring followed by higher spore densities in the summer of 2023. *Ceratonova shasta* spores were also detected later in the year, compared to 2022.

At the I-5 water sampling location, OSU first detected *C. shasta* in water samples on April 24 at less than one spore/liter. There was a spike in detection in early May at 13 spores/L and late May at 19 spores/L. However, spore concentration peaked at I-5 on June 20 at 67 spores/L (Oregon State University, 2023). This differs from 2022, when the first detection in water samples at I-5 occurred in early April and spore density was less than 20 spores/L for the entire year.

The pattern of low spore density in the spring was also observed at the Kinsman water sampling location. *Ceratonova shasta* was undetected in mid-April 2023, compared to 19 spores/L in 2022 for the same time period. Spore density in 2023 increased in early May to 22 spores/L and peaked on June 20 at 43 spores/L (Oregon State University, 2023). Again, this is a contrast to 2022, where spore density stayed below 20 spores/L at the Kinsman location.

Naturally produced Chinook Salmon

A total of 1,132 natural fish were collected in the Klamath River for testing by QPCR. Natural fish were collected the weeks of March 19 through June 4 in river reaches above the Trinity River confluence (K5, K4, K3, and K2). In 2023, fish collected from the Shasta River to Scott River (K4) reach were assayed in real-time for the first 11 weeks of the study (the week of March 19 to the week of May 28).

Ceratonova shasta was detected by QPCR in 26% (296/1,132, ci = 24-29%) of natural fish in 2023. The *C. shasta* POI has ranged from a low of 4% in 2012 to a high of 76% in 2014.

Ceratonova shasta POI in natural fish was highest in the Salmon River to Trinity reach (K2) at 52% (11/21, ci = 30-74%), and lowest in the Iron Gate Dam to Shasta River reach (K5) at 18% (63/358, ci = 14-22%).

Comparison of POI and DNA Copy Number in Natural Fish

Three logs or greater of *C. shasta* DNA is the threshold for describing *C. shasta* infections likely to lead to mortality under spring-summer water temperatures. For more information on how this threshold was determined, refer to Appendix A.

In the Iron Gate Dam to Shasta River (K5) reach, *C. shasta* was undetected during the first seven weeks of sampling. On week 8, the week of May 7, *C. shasta* POI was 7% (2/30, ci = 3-56%). The prevalence of infection increased to 70% (21/30, ci = 51-85%) in late May with one fish having greater than three logs of DNA (3.1 logs).

In the Shasta River to Scott River (K4) reach, *C. shasta* was undetected during the first six weeks of sampling. On week 7, the week of April 30, *C. shasta* POI was 37% (22/60, ci = 25-50%). The highest prevalence of infection occurred the week of May 28 (95%, 56/59, ci = 86-99%), which was the same week with the highest severity of infection. During the week of May 28, 29% of the weekly sample (17 fish) had greater than three logs of *C. shasta* DNA.

In the Scott River to Salmon River (K3) reach, nine weekly samples of natural fish were collected. *Ceratonova shasta* was undetected in week 3 through week 7. In week 8, POI was 15% (3/20, ci = 3-38%). Prevalence of infection increased to 95% (20/21, ci = 76-100%) during the week of May 21, and 14% of the weekly sample (3 fish) had greater than 3 logs of DNA.

Overall, *C. shasta* POI and severity were low in early spring. *Ceratonova shasta* POI and severity in natural fish were highest in late May for all reaches (K5, K4, and K3). Weekly POI and mean DNA copy number (log scale) from natural fish collected in the K4 and K3 reaches are depicted in Figure 3. Fish collected in the K5 reach are not included in this figure to compare 2023 to previous years (K5 sampling started in 2022).

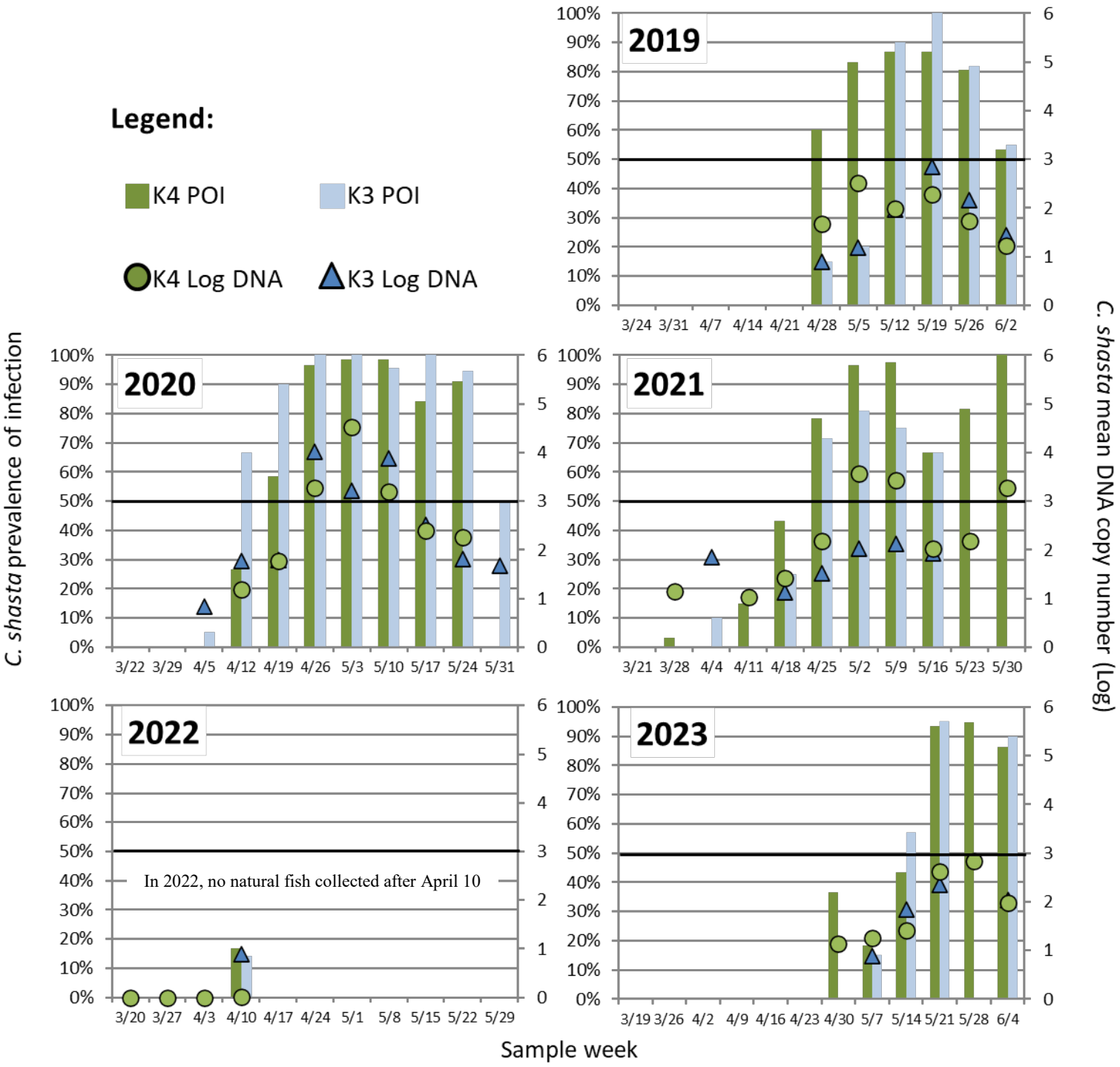

Figure 3. *Ceratonova shasta* prevalence of infection (POI) and weekly mean DNA copy number (log) in natural juvenile Chinook Salmon captured in the Shasta River to Scott River (K4) reach and the Scott River to Salmon River (K3) reach. Prevalence of infection shown in columns (Y-axis) and *C. shasta* mean DNA copy number (log) shown in circles and triangles (secondary Y-axis). Horizontal bold bar highlights 3 logs of *C. shasta* DNA threshold.

Gill Tissue Vs Intestinal Tissue by QPCR

A comparison of gill and intestinal tissue results was conducted in 2022 to investigate whether gill tissue is a more informative metric for real-time monitoring, and if *C. shasta* detection from gill tissue can provide managers with more timely information about the onset of infection. The testing of two tissues from the same individual fish continued in 2023 and was conducted only on natural origin fish collected at the Kinsman RST.

Both tissue types were tested by QPCR starting the week of March 19 through the week of May 28 (11 weeks total). *Ceratonova shasta* was not detected by QPCR in gill or intestinal tissue for the first six weeks of the season (Figure 4). On week 7, the week of April 30, *C. shasta* was detected in both tissue types. The prevalence of infection was higher in intestinal tissue (37%, 22/60, ci = 25-50%) compared to gill tissue (12%, 7/60, ci = 5-23%) tested that week. During the next two weeks, weeks 8 and 9, *C. shasta* POI was higher in gill tissue compared to intestinal tissue.

In late May, however, *C. shasta* POI was higher in intestinal tissue. More importantly, the number of severe infections increased during these two weeks. Severe infections were observed in both gill and intestinal tissue. In weeks 10 and 11, 6% of gill tissue was over 3 logs of DNA, compared to 27% in intestinal tissue. These results indicate that *C. shasta* POI was high (93-95%) but there was also a divergence towards to severe infection in the intestine during late May. According to weekly abundance estimates conducted by the Arcata Fish and Wildlife Office, approximately 45% of natural origin Chinook Salmon had migrated downstream of the Kinsman site by the end of May (personal communication with Steve Gough, USFWS). That indicates that the majority of natural origin fish were still in the upper river when POI in intestinal tissue was high and spore concentrations at Kinsman were increasing.

The prevalence of infection in fish tissues was overlaid with spore density data from OSU (Figure 4). *Ceratonova shasta* was detected above one spore/L at the Kinsman index site during the week of April 23 (Oregon State University, 2023). Spore density climbed quickly in the following weeks and peaked at Kinsman at 22 spores/L during the week of May 7 (Oregon State University, 2023). The prevalence of infection in gill tissue during late April and early May was also increasing. In both 2022 and 2023, OSU spore data and gill POI tracked each other well during the onset of infection.

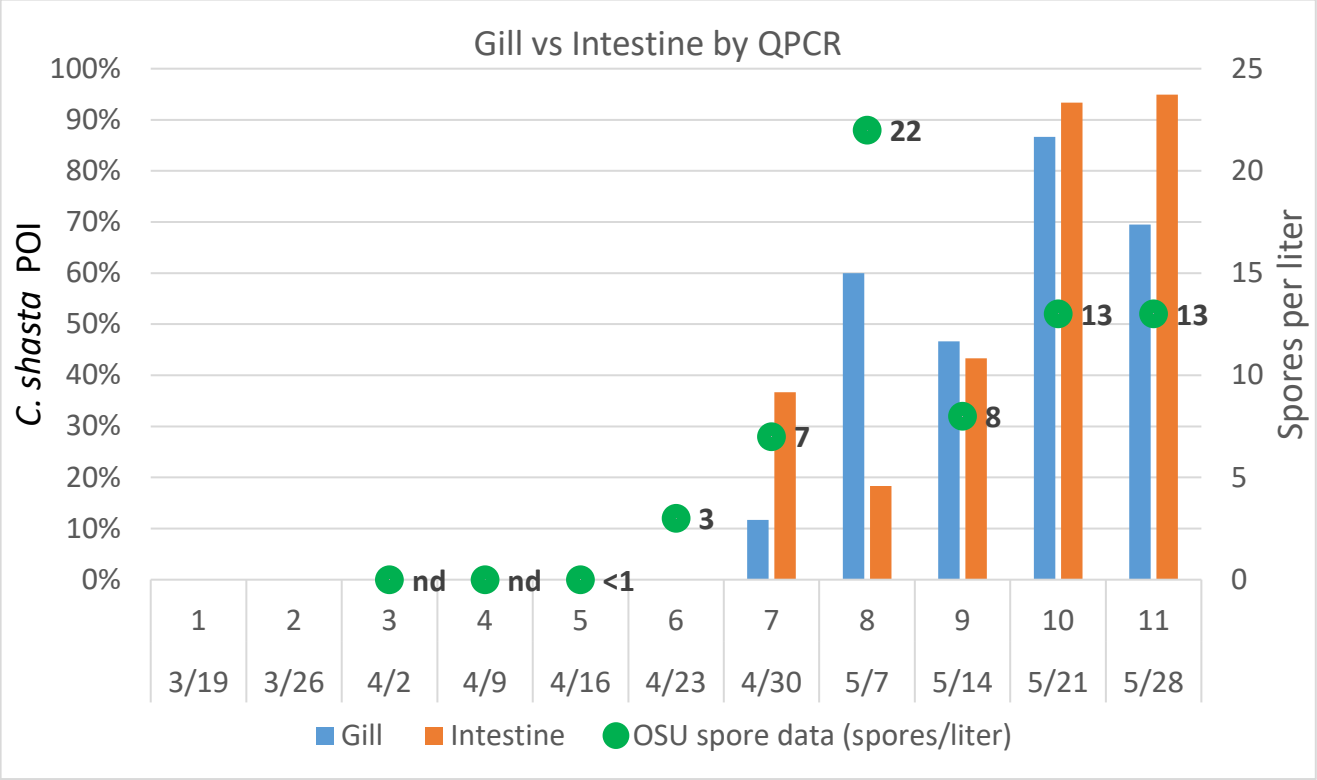

Figure 4. Weekly QPCR results for fish collected at Kinsman RST during real-time monitoring. *Ceratonova shasta* POI by QPCR is shown in bar graph for gill tissue (blue) and intestinal tissue (orange). Weekly water sampling results from Oregon State University is shown with a green dot and the number of spores/L. nd = water samples tested but *C. shasta* was not detected.

Gill tissue may be a more informative metric than intestinal tissue for real-time monitoring because it is a snapshot of exposure when the sample is collected. Intestinal tissue results represent a fish that was previously exposed and time is required for the parasite to be detected in the intestine. Past studies have shown that *C. shasta* can be detected in intestinal tissue 4 to 5 days post-exposure (Bjork and Bartholomew 2010, True et al., 2012), however, parasite density and water temperatures could affect that timing. *Ceratonova shasta* POI in gill tissue may provide real-time information on infection status, compared to intestinal tissue which represents prior exposure.

Water sample data from OSU paired with gill tissue results by QPCR may be a predictor of *C. shasta* POI in future weeks. Unfortunately for this analysis, we had fewer weeks to compare gill and intestinal tissue in 2023 due to no infection in late March and the majority of April. This topic warrants more discussion with basin cooperators to determine if sampling gill tissue should be used as the primary metric of real-time POI.

Chinook Salmon collected by RST and assayed for *C. shasta* by QPCR

A comparison of fish collected using rotary screw traps on the main-stem Klamath River was not conducted in 2023. Due to high flow conditions, the Weitchpec rotary screw trap could not safely be installed until late June (personal communication with Leanne Knutson, Yurok Tribe Fisheries Department). Therefore, even though there was a higher proportion of hatchery fish collected and tested in 2023, we were unable to track a cohort of fish past all rotary screw trap locations.

Unknown Chinook Salmon

A total of 234 fish of unknown origin were collected from the week of June 11 to July 25. *Ceratonova shasta* was detected by QPCR in 76% (179/234, ci = 71-82%) of unknown origin Chinook Salmon, and 15% (n = 36) of the fish had greater than 3 logs of *C. shasta* DNA.

Ceratonova shasta POI was highest for unknown origin fish collected in the Shasta River to Scott River (K4) reach at 88% (38/43, ci = 12-22%). Prevalence of infection for unknown origin fish was lowest at 30% (6/20, ci = 12-54%) in the upstream reach from Iron Gate Dam to Shasta River (K5).

Iron Gate Hatchery (CWT) Chinook Salmon

Iron Gate Hatchery released ~4.7 million fall-run Chinook Salmon smolts over three days in June 2023 (personal communication with Patrick Brock, CDFW).

Coded-wire tagged salmon originating from IGH were collected in the Klamath River from June 13 to August 16. *Ceratonova shasta* was detected in 66% (281/424, ci = 32-71%) of IGH CWT screened by QPCR. Prevalence of infection for *C. shasta* was highest at 97% (143/148, ci = 92-99%) in the Salmon River to Trinity River (K2) reach. Prevalence of infection was lowest in the Iron Gate Dam to Shasta River (K5) reach at 19% (7/37, ci = 8-35%).

IGH CWT Weeks At Large

Weeks at Large (WAL) analysis has not been conducted since 2019 because the number of IGH smolts recaptured in-river has been low in recent years. For more information and the definition of Weeks at Large, refer to Appendix A.

In 2023, the highest *C. shasta* prevalence of infection occurred in fish residing 3 WAL (94%, Figure 5). Hatchery fish collected in the river from 1 WAL to 5 WAL had *C. shasta* POI greater than 70%. In 2023, low *C. shasta* prevalence of infection occurred in fish residing 7 WAL (26%). The graph depicts prevalence of infection increasing quickly once IGH fish were in the Klamath River and the POI remained high for many weeks.

It is worth noting that *C. shasta* was detected in 27% of CWTs that were in the Klamath River for less than one week. That is a short amount of time for the parasite to be detected in the intestine and is indicative of a high exposure dose. Fish <1 WAL were captured in mid to late June when spore concentrations were between 43 and 61 spores/liter at the upper index sites (Appendix E, Oregon State University, 2023) and the mean daily water temperature at the gauge below IGD ranged from 17.7°C to 19.4°C.

In IGH CWT Chinook Salmon, the mean DNA copy number peaked at 2.79 logs during 2 WAL. DNA copy number remained high the following week at 2.75 logs (3 WAL). These results indicate that a larger proportion of Chinook in 2-3 WAL were infected when recaptured and the severity of infection was also the highest in hatchery fish this season. This observation complements the OSU spore data since fish residing 2 WAL were recaptured in late June and early July, when spore densities at OSU index sites were increasing.

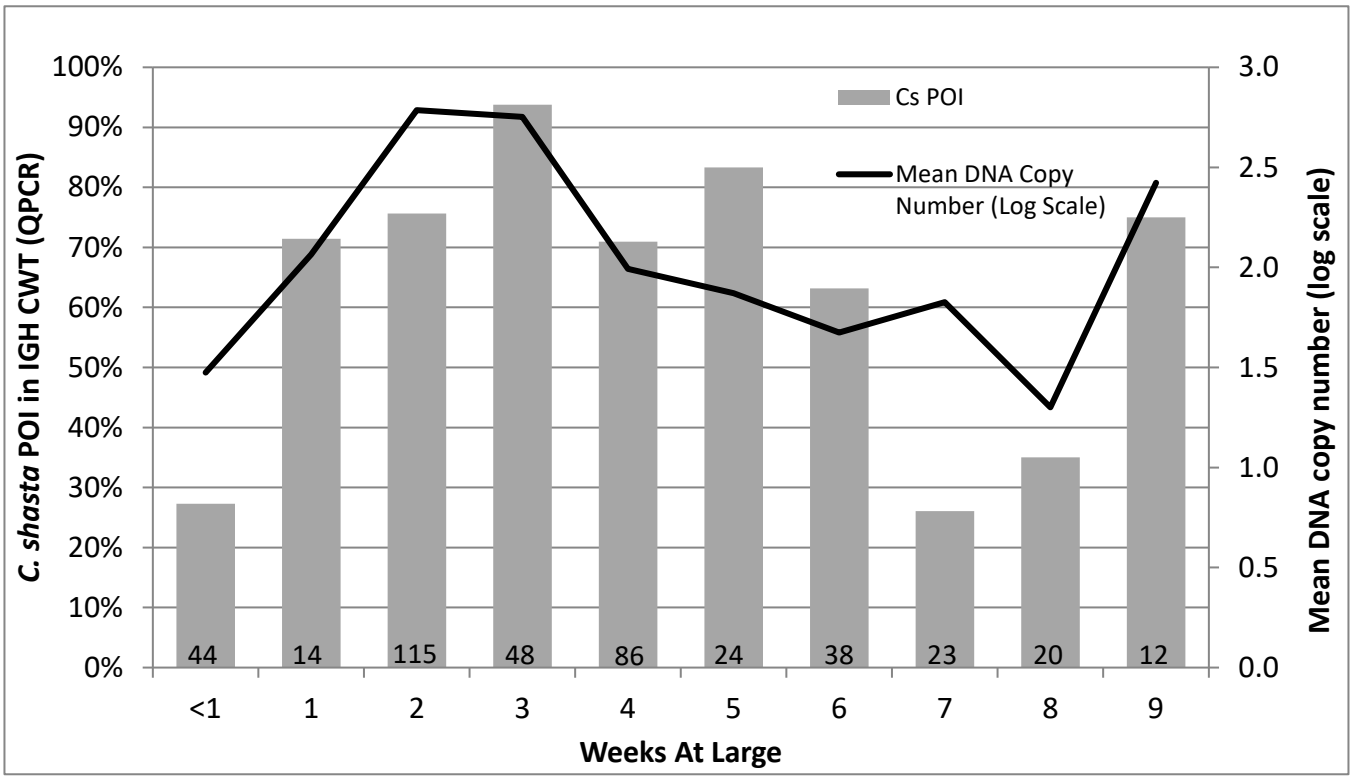

Figure 5. *Ceratonova shasta* prevalence of infection in IGH CWT intestinal tissue by Weeks At Large (WAL) post hatchery release. The bar graph is prevalence of infection (%) on the primary y-axis and the line graph is the mean *C. shasta* DNA copy number (log scale) on the secondary y-axis for Chinook Salmon tested by QPCR. The number of fish collected is listed inside the base of each bar.

Ceratonova shasta was undetected in 83 CWTs collected in the estuary; that included fish in the river for 1-9 WAL. One individual fish that was released from IGH on June 20 migrated quickly and was recaptured at the Estuary sampling site (Glen site, below McGarvey Creek) on June 28. The majority of uninfected IGH smolts in the Estuary reach had been in river for 2 WAL. This might indicate that migration timing was important in 2023. Hatchery fish that out-migrated quicker may have avoid the time period when spore concentration was highest (late June through August). There were three individual hatchery fish that were in the Klamath River for a longer time period (9 WAL) and tested negative for *C. shasta* by QPCR when recaptured in the Estuary.

Historical Comparison

Prevalence of *C. shasta* infection during the peak out-migration by QPCR increased in 2023, relative to previous years (Table 2.) Prevalence of *C. shasta* infection by QPCR during this period was 62% (665/1,066, ci = 59-65%) in 2023, compared to 53% in 2022. *Ceratonova shasta* POI was higher than the average of 51% for the past fifteen years (2009-2023).

Table 2. Historic annual *Ceratonova shasta* POI in all juvenile Chinook Salmon collected from the main-stem Klamath River between Iron Gate Dam and Trinity River confluence during the peak out-migration period of May through July 2009-2023. Percent positive by assay is reported, as well as the number positive/number tested in parenthesis.

Year	Histology (% Positive)		QPCR (% Positive)	
2009	54%	(50/93)	47%	(264/561)
2010	15%	(22/146)	17%	(128/774)
2011	3%[1]	(3/118)	17%	(62/374)
2012	9%	(9/98)	30%	(160/526)
2013	16%	(6/37)	46%	(234/508)
2014	42%	(20/48)	81%	(467/576)
2015	62%	(37/60)	91%	(437/482)
2016	14%	(8/58)	48%	(243/504)
2017	8%	(3/40)	26%	(153/600)
2018	4%	(1/27)	20%	(114/570)
2019	40%	(16/40)	68%	(395/581)
2020	60%	(18/30)	73%	(433/593)
2021	75%	(24/32)	82%	(368/447)
2022	32%	(16/50)	53%	(472/896)
2023	No histology testing in 2023		62%	(665/1066)
Mean	**27%**	**(233/877)**	**51%**	**(4,595/9,058)**

[1] Histology performed in K4 and K1 reach in 2011. From 2012 to 2022 histology was performed in K4 and K3 reach.

Environmental Conditions

California's three driest years of record (Water Years 2020-2022) were followed by a wet and snowy 2023. Water Year 2023 was also notably cooler statewide compared to recent years. (CA Department of Water Resources, 2023). Detailed figures of water temperature and Klamath River discharge are provided in Appendix D.

Observations and Questions in 2023

- The onset of infection occurred later in 2023 (early May) compared to 2022 (mid-April). OSU spore density results in water samples also increased later in the year and were of higher magnitude, compared to 2022 (Oregon State University, 2023).
- In late May, natural fish collected from Kinsman had high *C. shasta* POI (93-95%, intestinal tissue). Approximately 45% of natural fish had migrated downstream of Kinsman at this time, indicating that the majority of natural fish were still located in the upper river when POI was high and spore densities were increasing.
- *C. shasta* was detected in the intestine of Iron Gate Hatchery fish recaptured <1 WAL. It was surprising the infection was detected so quickly in intestinal tissue, but the spore density was high in the upper river reaches in late June.
- Why did the historic *C. shasta* POI (May-July above the TR) increase in 2023 when environmental conditions were favorable? Water Year 2023 was wetter and river discharge was higher in 2023 below IGD and at Seiad Valley compared to 2022. Water temperatures were cooler in the spring, but warmer in May, June, and July below IGD. Perhaps warmer summer water temperatures influenced the POI increase we observed.

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Eric Reiland, BOR

Photo contributions: Anne Voss, USFWS
Cover Photo: Klamath River, CA-NV Fish Health Center

Author Roles

The contributions of each author have been summarized below.

- Anne Voss – Project lead and real-time data analysis, data management and quality control, pivot tables, and addressing reviewer comments. Primary author for 2023 annual report.
- Ron Stone – Sampling coordination, necropsy, DNA extraction, QPCR assays, data entry, and report writing.
- Scott Freund – Necropsy assistance, CWT extraction and reading

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Appendix A - Definitions and Data Analysis

Testing

In 2023, tissues were tested for *C. shasta* by QPCR only. Histology testing was not conducted in 2023. *Parvicapsula minibicornis* was not tested in 2023.

Real-time Monitoring

Real-time monitoring provided timely data to water resource managers during juvenile Chinook Salmon out-migration. Data is provided in real-time during April and May. The goal was to provide estimates of *C. shasta* POI and DNA copy number by QPCR to managers weekly. Real-time monitoring occurred in the Shasta River to Scott River reach (K4). The Kinsman RST was the collection site for real-time monitoring.

The Kinsman RST was deployed at the beginning of a sample week for fish collection. Field crews collected fish from the trap, euthanized fish, bagged fish with collection tags, and stored frozen. Coordination for sample transfer to the Fish Health Center was done as soon as fish became available, typically on Tuesday or Wednesday of a sample week. The Fish Health Center required that fish be transported to the laboratory no later than Wednesday afternoon for real-time results to be reported that week. Necropsy and DNA extraction were completed mid-week, QPCR assay and analysis later in the week, and results were reported on Friday.

Prevalence of Infection and Copy Number

Point prevalence of infection and annual prevalence (defined by Durfee, 1978; USFWS, 2022) for *C. shasta* is reported with 95% confidence intervals (denoted ci) due to sample size. The larger the sample size, the smaller the confidence interval. Confidence interval calculations were done in Excel as follows:

Prevalence of infection (POI) is used to describe the proportion of infected Chinook salmon (numerator) in the sample (number of animals examined) for a particular calendar week. Annual prevalence is used to describe the overall prevalence of infection in the sampled population during the entire sampling period that year. Annual prevalence estimates are not estimates of the annual proportion of the population that is infected, because weekly estimates are not weighted by abundance values.

DNA copy number is a term used to describe the quantity of parasite DNA within fish tissue. The QPCR assay quantitates unknown samples based on a known quantity from a standard curve. DNA copy number is reported in log scale. The log value is calculated by transforming the DNA copy number for an individual fish to log scale first and then taking the mean of the log values for that group of fish. Unlike water samples that relate DNA concentration to actinospore number, tissue samples contain various multinucleated parasite developmental forms (trophozoites, pansporoblast) that have not been empirically correlated to DNA concentration.

Comparison of POI and DNA Copy Number in Natural Fish

To assess *C. shasta* disease in out-migrating juvenile fish, it is important to look at both prevalence of infection, as well as DNA copy number. As stated above, POI is the proportion of fish infected; however, POI alone does not provide the entire picture of infection status. For example, a high weekly POI suggests that a large proportion of fish are infected, but provides no context as to whether fish are in a disease state. It is informative to also consider DNA copy number (how much parasite DNA is in the fish tissue) to get a more complete picture of infection.

Three logs or greater of *C. shasta* DNA is the threshold for describing *C. shasta* infections likely to lead to mortality under spring-summer temperatures. This threshold was based on a sentinel exposure study conducted in 2008 (True et al. 2012) and additional samples collected in 2020 and 2021 (Voss et al. 2022). The relationship between copy number and histological rating of tissue for *Ceratonova shasta* infection in the intestine was determined by dividing tissues from a single fish and testing by both QPCR and histology. There was good agreement between histological signs of disease and QPCR values 3 logs or greater.

Weeks At Large Analysis

In the monitoring program, temporal data is derived from IGH CWT codes obtained from juvenile Chinook Salmon with known exposure periods (hatchery release to in-river recapture date). The period of how long fish reside in the Klamath River post-hatchery release is termed Weeks At Large (WAL). For example, fish captured six days or less after hatchery release would be classified as <1 WAL. Fish captured between 7 and 13 days would be classified as 1 WAL, fish captured between 14 and 20 days would be 2 WAL, etc.

In 2020-2022, the Weeks At Large analysis was not conducted. In 2020 and 2022, too few hatchery fish were recovered and the sample size was too small. In 2021, the California Department of Fish and Wildlife retained juvenile salmon within its hatchery system over the summer until Klamath River conditions improved in the fall.

Historical Comparison

Prevalence of infection by QPCR is the metric that has been used for historical comparisons of disease prevalence in the monitoring program since 2009. Data are confined to the peak migration timing of May 1 to July 31 (USFWS 1997) and fish of any origin collected above the Trinity confluence.

Appendix B – Methods for *Ceratonova shasta* Quantitative PCR Assay

A *Ceratonova shasta* quantitative PCR assay targeting the 18S ribosomal DNA sequence was used to assay DNA extracted from fish tissues. Forward primer (Cs-1034F 5' CCA GCT TGA GAT TAG CTC GGT AA), reverse primer (Cs-1104R CCC CGG AAC CCG AAA G), and probe (CsProbe-1058T 6FAM CGA GCC AAG TTG GTC TCT CCG TGA AAA C TAMRA) sequences were used from Hallett et al., 2006.

All reactions (30µL) were conducted in a 96-well optical reaction plate using 0.9 µM of both primers, 0.25 µM of probe, 15 µL of TaqMan Universal PCR Master Mix, and 5uL of DNA template. Samples were not assayed in replicates. Reactions were assayed using a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. 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.

The *C. shasta* reference standard curve was obtained using synthesized DNA (gBlock Gene Fragments, Integrated DNA Technology, 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 ten orders of magnitude in Tris-EDTA (ethylenediamine tetraacetic acid) buffer. Using QPCR analysis software, the cycle threshold (C_T) values for each standard concentration were calculated (QuantStudio 6 software, v 1.7.2, Applied Biosystems). The standard curve was used to evaluate PCR amplification efficiency (slope of the standard curve, efficiency was 97%), fit to the curve (R^2 value = 0.999), and the y-intercept (38.4) which is the theoretical C_T value for a single copy of parasite DNA when assays are 100% efficient (Applied Biosystems, 2014).

Each assay was evaluated for expected C_T values of the reference standards and assay efficiency. At the end of the season, any plates with more than a 3% decrease in assay efficiency from the mean were retested and reevaluated. Based on this criteria, one plate (gill tissue only) did require retesting in 2023.

Our criteria for a positive test result required samples to produce a change in normalized fluorescent signal (Δ Rn) greater than or equal to 100,000 florescent units, verifying significant amplification above background levels of the instrument. Samples were also required to have a quantity (DNA copy number) greater than or equal to five copies, as very low copy numbers are not reliable given the limitations of the Poisson distribution (Applied Biosystems, 2016).

In 2022, it was decided in that auto-threshold and auto-baseline settings would be used for analysis on the QuantStudio 6 instrument software. These auto settings will be used as long as the reaction efficiencies are similar between experiments (within 3%) and efficiencies are between 90% and 110%. These analysis settings will also be used in future years of *C. shasta* testing on the QuantStudio 6 instrument.

Appendix C – QPCR Results

Table C-1. *Ceratonova shasta* infection by QPCR in juvenile Chinook salmon sampled from six reaches within the Klamath River.
The prevalence [% (number positive/number tested)] is presented for each sample reach by collection week and sample date (Sunday of each week).

Week	Sample Date	IGD to Shasta R. (K5)	Shasta R. to Scott R. (K4)	Scott R. to Salmon R. (K3)	Salmon R. to Trinity R. (K2)	Trinity R. to Estuary (K1)	Estuary (K0)
1	19-Mar	0% (0/30)	0% (0/18)				
2	26-Mar	0% (0/30)	0% (0/30)				
3	2-Apr	0% (0/28)	0% (0/59)	0% (0/20%)			
4	9-Apr	0% (0/30)	0% (0/43)	0% (0/20%)			
5	16-Apr	0% (0/30)	0% (0/60)	0% (0/22%)			
6	23-Apr	0% (0/30)	0% (0/58)	0% (0/22%)			
7	30-Apr	0% (0/30)	37% (22/60)	0% (0/20%)			
8	7-May	7% (2/30)	18% (11/60)	15% (3/20)			
9	14-May	30% (9/30)	43% (26/60)	57% (12/21)			
10	21-May	70% (21/30)	93% (28/30)	95% (20/21)			
11	28-May	40 (12/30)	95% (56/59)				
12	4-Jun	63% (19/30)	86% (25/29)	90% (19/21)	52% (11/21)		
13	11-Jun	26% (7/27)[A]	82% (23/28)[B]	80% (16/20)	27% (12/21)[C]		
14	18-Jun	20% (6/30)[B]	86% (25/29)[B]	64% (14/22)	90% (37/41)[B]		
15	25-Jun			81% (17/21)[A]	100% (42/42)[A]	67% (4/6)[C]	14% (4/29)[A]
16	2-Jul			79% (15/19)[A]	98% (41/42)[B]		39% (9/23)[A]
17	9-Jul			90% (19/21)[A]	100% (40/40)[B]		64% (9/14)[A]
18	16-Jul			89% (17/19)[A]	95% (19/20)[A]	80% (12/15)[C]	100% (6/6)[A]
19	23-Jul				86% (19/22)[B]	79% (15/19)[C]	42% (5/12)[A]
20	30-Jul			10% (1/10)[A]	78% (7/9)[A]		0% (0/11)[A]
21	6-Aug						25%(5/20)[A]
22	13-Aug						70% (14/20)[A]
		K5 Total **18% (76/415)**	K4 Total **35% (216/623)**	K3 Total **48% (153/319)**	K2 Total **88% (228/258)**	K1 Total **78% (31/40)**	K0 Total **39% (52/135)**

[A] Weekly sample contains CWTs only [B] Weekly sample contains both unmarked fish and CWTs [C] Weekly sample contains unknown fish only

Appendix D – Environmental Conditions

River Water Temperature

Water temperatures in early spring were cooler in 2023, compared to 2022, below Iron Gate Dam. In 2023, water temperatures below Iron Gate Dam started low in early March (approximately 4ºC) and climbed steadily to 13.7ºC on April 30. Temperatures remained around 13°C through May 8 then continued to rise throughout the month (approximately 19°C on May 31). There were three time periods during June when temperatures fell slightly, 18.4°C, 18.2°C, and 17.7°C respectively. Temperatures continued to rise from the end of June through early July, approximately 18°C-21°C and remained high through late August (~21°C). Below Iron Gate Dam mean daily water temperature peaked at 21.45ºC on July 26 (Figure D-1).

In previous study years, we typically observed mean daily water temperatures of approximately 18ºC, and often as high as 23ºC below Iron Gate Dam, during the peak juvenile migration period of May through July. In 2023, the mean daily water temperature was 18.3ºC (ranging from 12.9–21.5ºC) during this period.

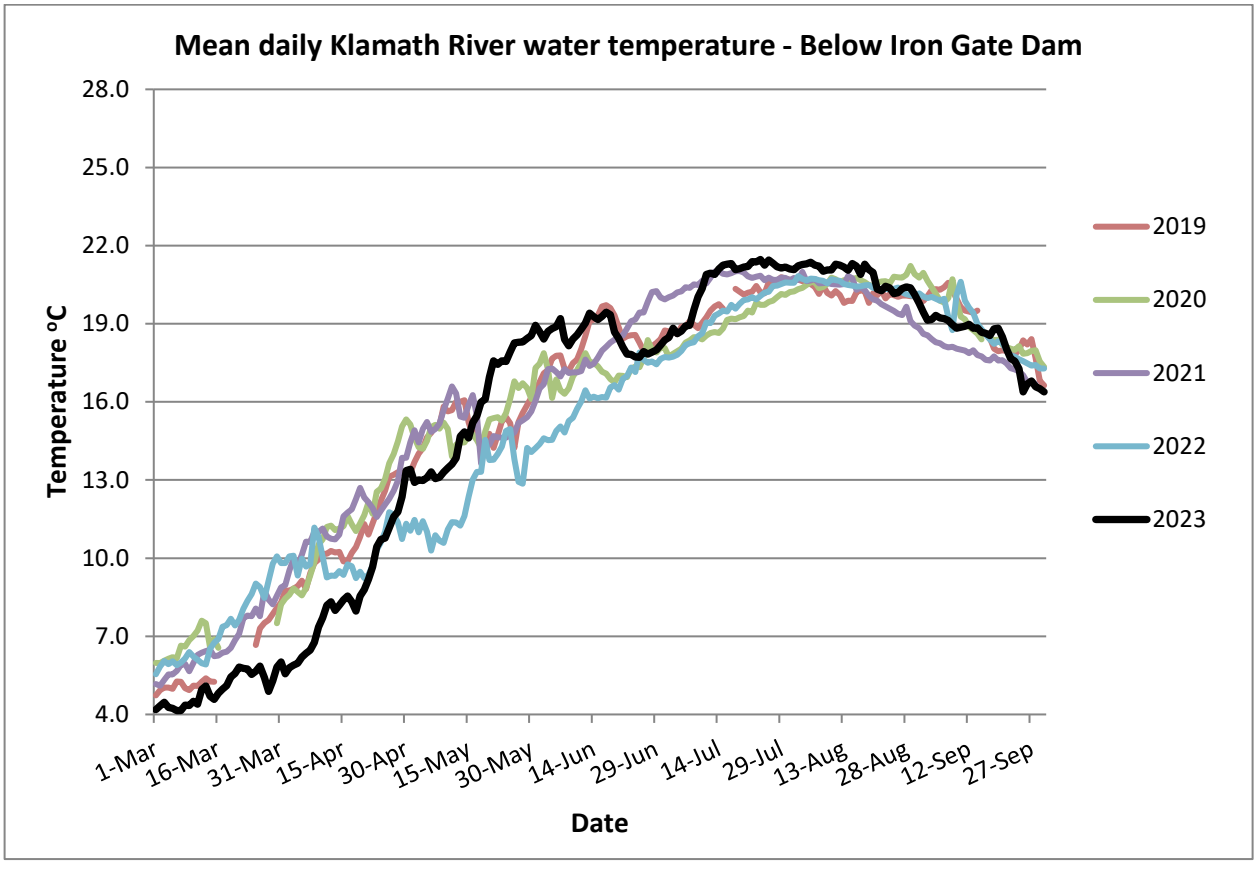

Figure D-1. Mean daily Klamath River water temperature below Iron Gate Dam from March through September 2019-2023. Temperature data was acquired from the Karuk Tribe.

Another temperature gauge is located in the Scott River to Salmon River (K3) reach, near Seiad Valley. Unlike the water temperatures below Iron Gate Dam, this gauge has less influence from the dam, and the water temperatures are more variable.

Water temperatures at Seiad Valley were approximately 6.0ºC in March and climbed to 12.2ºC on April 27. Water temperatures were variable April through July however, temperatures during this period trended upward starting with a low of 6.9°C in April and ending with a high of 25.6°C in July. Temperatures from July through mid-August were very similar with mean daily temperatures of 23.8°C and 23.4°C respectively. Maximum daily temperatures during the same time period were also extremely similar, 25.6°C and 25.9°C respectively. The mean daily water temperature at Seiad Valley peaked at 25.9ºC on August 15. Water temperatures at Seiad Valley were 0.5ºC to 4.0ºC cooler in early spring and late summer of 2023, compared to 2022 (Figure D-2).

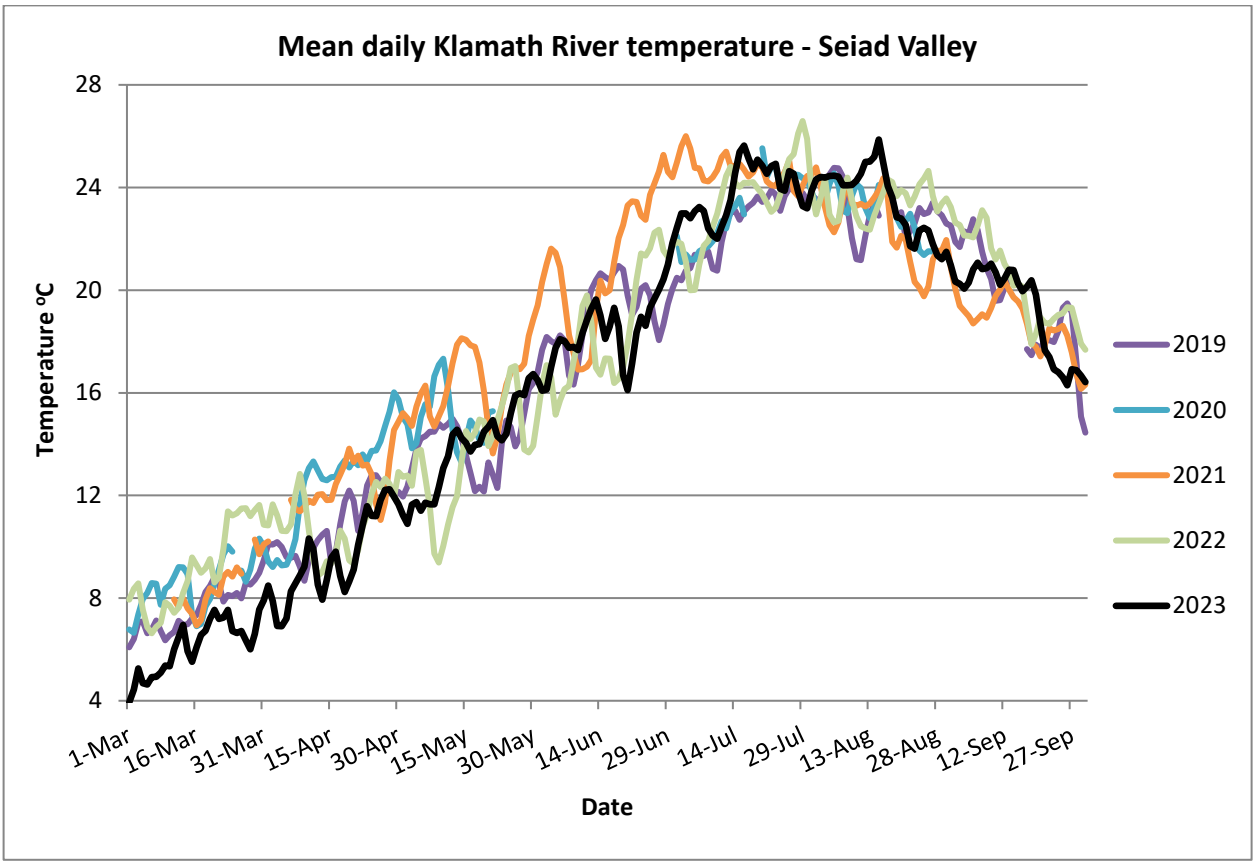

Figure D-2. Mean daily Klamath River temperature at Seiad Valley from March through September 2016-2023. Temperature data was acquired from the Karuk Tribe.

In previous study years, we typically observed mean daily water temperatures of approximately 19ºC, and often as high as 25ºC at Seiad Valley, during the peak juvenile migration period of May through July. In 2023, the mean daily water temperature was similar to the 2022 season during peak juvenile out-migration. Temperatures at Seiad Valley averaged 18.7ºC (ranging from 10.9 – 25.6ºC) during this period.

River Discharge

Discharge was fairly consistent (973 – 1,920 cfs March-September) except for a modified surface flushing flow event in mid-April. Bureau of Reclamation (BOR) began increased flows at Link River Dam (LRD) and below Iron Gate Dam (IGD) to reduce the risk of disease on April 19 – 22. Flows at Link River Dam were increased to 5,300 cfs and 6,030 cfs out of IGD (Bureau of Reclamation 2023). Peak discharge lasted for 72 hours. Discharge was above 2,000 cfs for 24 days (April 19-June 2) with the peak occurring on April 20 at 6,140 cfs (Figure D-3). Discharge decreased below 1,000 cfs on July 1 and the minimum discharge observed during the sampling season was 896 cfs on July 16.

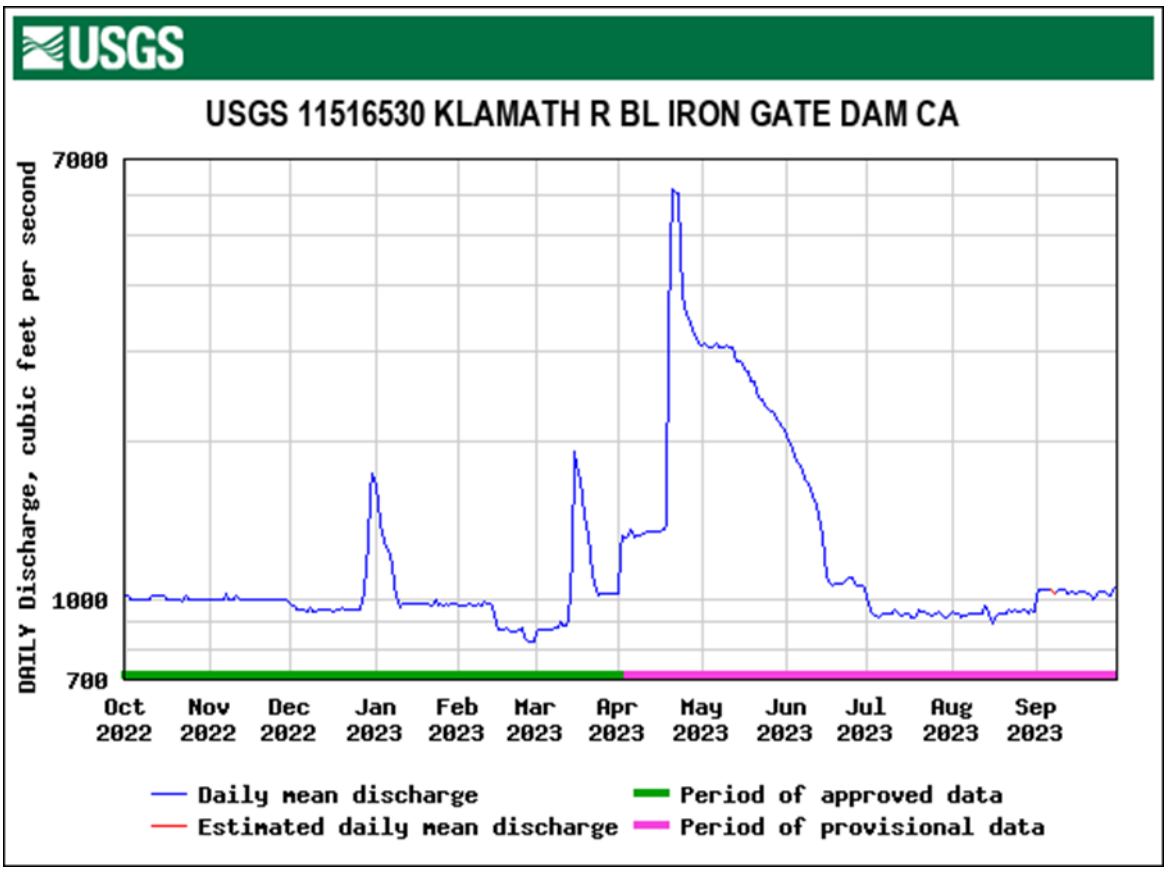

Figure D-3. Daily mean discharge (cfs) below Iron Gate Dam for Water Year 2023 (October 1, 2022 through September 30, 2023. Data was collected from USGS gaging station 11516530 at waterdata.usgs.gov.

As is the case with water temperature, the hydrograph from Seiad Valley shows greater variability over the summer, compared to discharges below IGD. Discharge was above 1,000 cfs throughout the 2023 sampling season. The peak discharge at Seiad Valley was 9,230 cfs on April 30 and again that was associated with a modified surface flushing flow event (Figure D-4). The minimum discharge observed during the sampling season was 1,030 cfs on August 17.

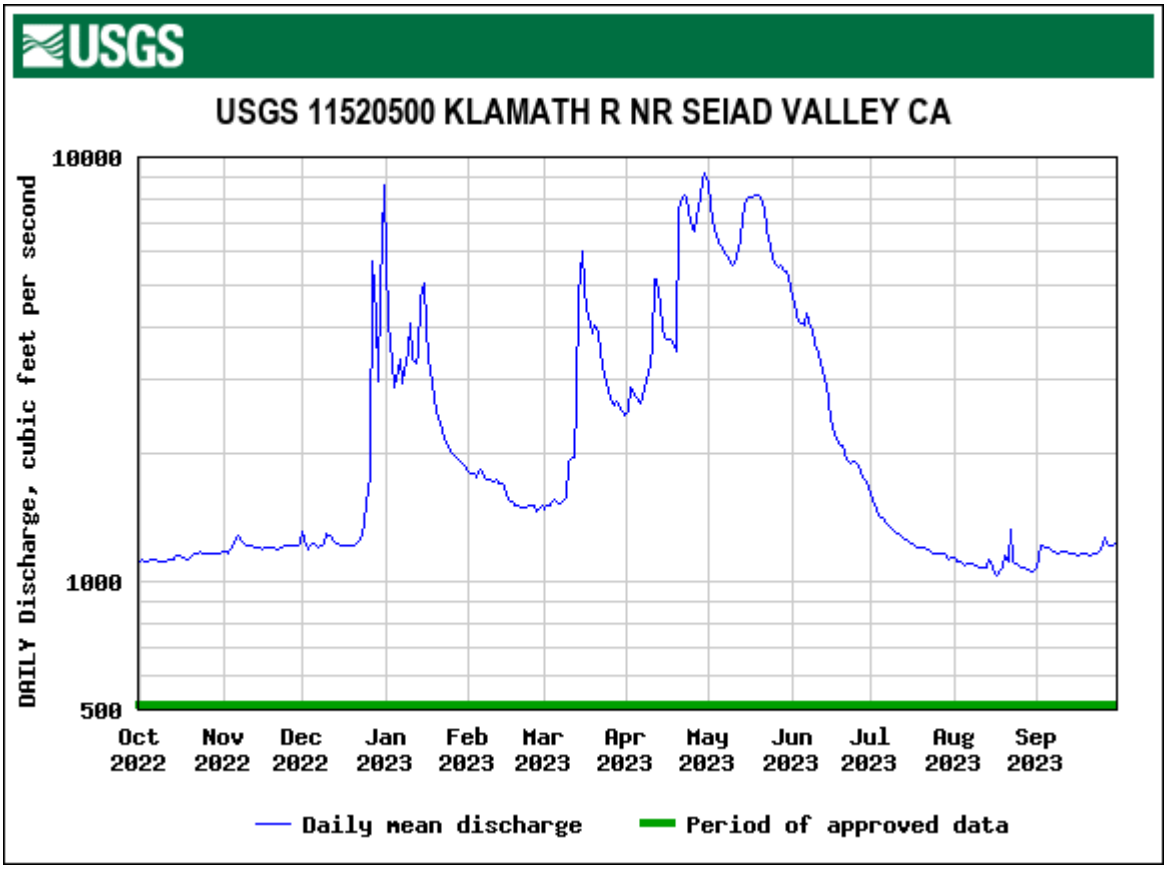

Figure D-4. Daily mean discharge (cfs) near Seiad Valley for Water Year 2023 (October 1, 2022 through September 30, 2023. Data was collected from USGS gaging station 11520500 at waterdata.usgs.gov.

Daily river discharge below Iron Gate Dam for the last fifteen years is shown in Figure D-5. Both 2014 and 2015 were ranked as extreme drought years. In 2017 discharge remained above 2,000 cfs for most of the spring and summer, as 2017 was a wet year. Discharge was low in 2018, with most of the year at less than 2,000 cfs except for the peaks when the U.S. Bureau of Reclamation released water. Discharge in 2022 was similar to 2021, where the majority of the year the discharge was recorded close to 1,000 cfs. Flows during the 2023 sampling season (March-August) averaged 1,507 cfs. The highest flows occurred in April following a modified surface flushing flow over a 72 hours, 6,500 cfs. Discharge averaged over 1,000 cfs in March-July and again in September. July and August were similar averaging 934 cfs and 938 cfs respectively (Bureau of Reclamation 2023).

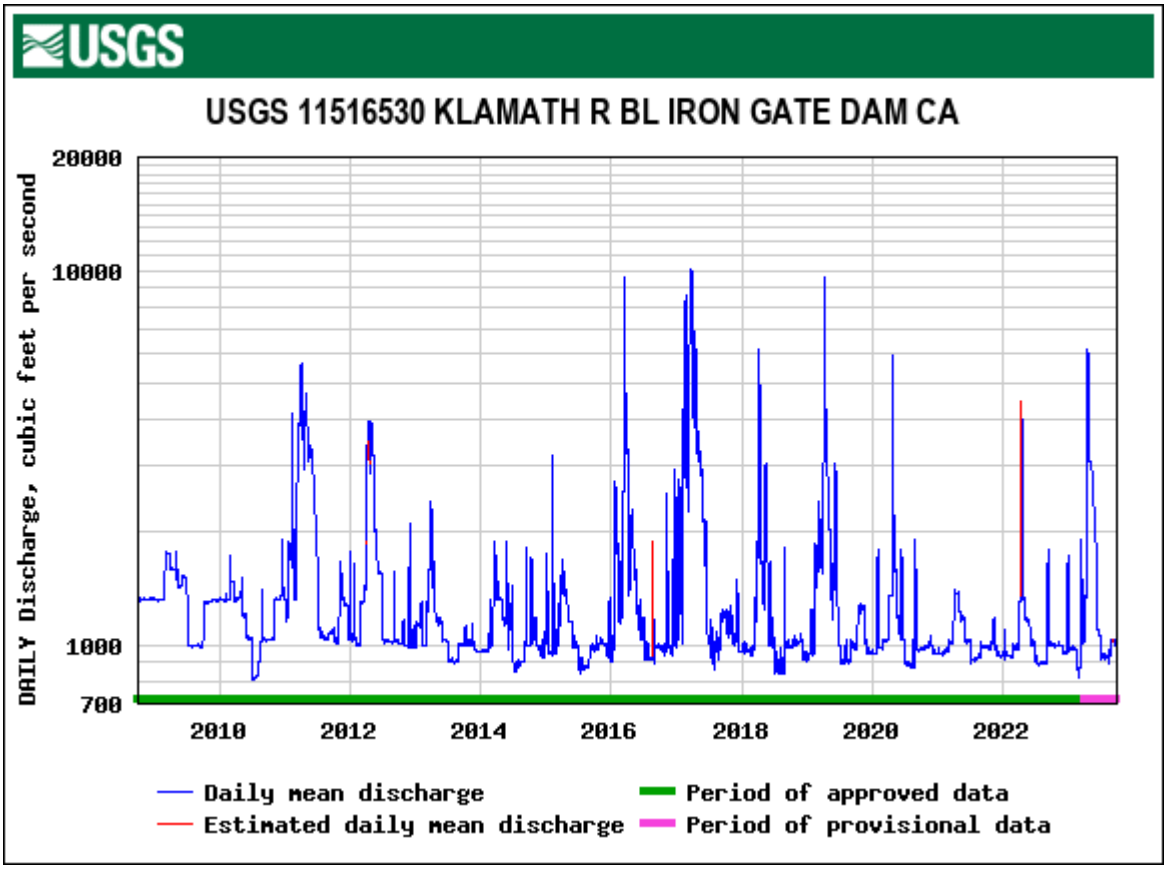

Figure D-5. Daily discharge (cfs) below Iron Gate Dam from October 2008 through September 2023. Data acquired from USGS waterdata.usgs.gov

Appendix E – Spore Density

Dr. Sascha Hallet from Oregon State University monitors and quantifies the waterborne stages of *C. shasta* from Klamath River water samples.

More information can be found on the OSU monitoring website:
https://microbiology.oregonstate.edu/research/aquatic-microbiology-ecology/monitoring-studies

Below is a figure from OSU showing the *C. shasta* average spore density in water samples during 2023.

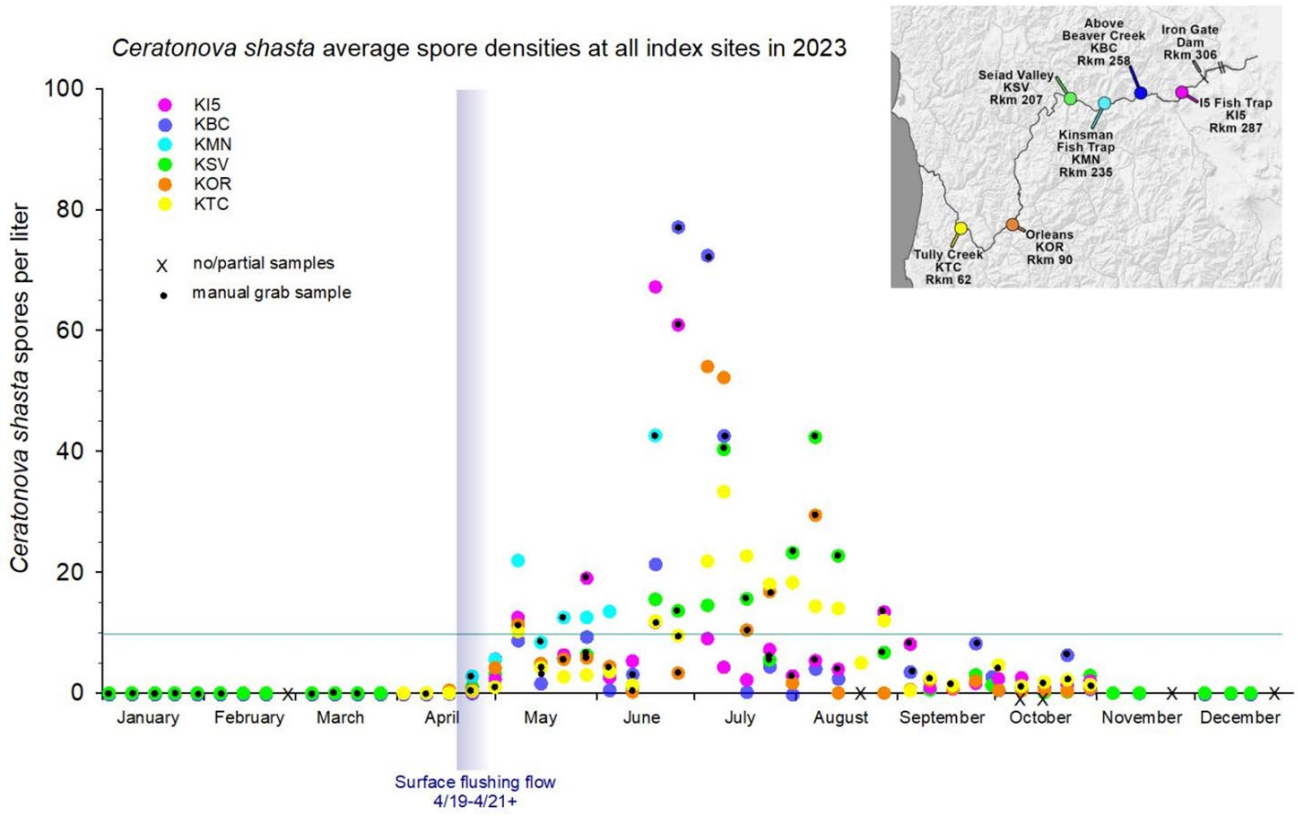

Figure E-1. Density (average spores per liter) of Ceratonova shasta in 24-hour composite water samples collected at the mainstem index sites in 2023. Note that KMN is sampled only during salmonid outmigration, KBC and KSV year round and remaining sites April through October. KBC = near Beaver Creek, KSV = Seiad Valley, KI5 = near I5 bridge, KTC = Tully Creek, KMN = Kinsman Fish Trap, KOR = Orleans. The line denotes 10 spores per liter which corresponds with 40% mortality threshold in Chinook salmon. A managed surface flushing flow event commenced April 19th (6030 cfs for 3 days).