U.S. Fish & Wildlife Service

California-Nevada Fish Health Center Investigational Report:

Myxosporean Parasite (*Ceratonova shasta* and *Parvicapsula minibicornis*) Prevalence of Infection in Klamath River Basin Juvenile Chinook Salmon, March – July 2020

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Summary

Juvenile Klamath River Chinook Salmon (*Oncorhynchus tshawytscha*) were assayed from late March through July 2020 by quantitative polymerase chain reaction (QPCR) and histology for myxosporean parasite infection of *Ceratonova shasta* and *Parvicapsula minibicornis*. The annual prevalence of *C. shasta* infection in 2020 by QPCR was 61%. The majority of fish in 2020 were coinfected with both *C. shasta* and *P. minibicornis.*

Natural fish were monitored in real-time for the first nine weeks of the season in order to provide timely data to fishery managers. *Ceratonova shasta* was first detected in fish sampled on April 14 (week four) in the Shasta River to Scott River reach. *Ceratonova shasta* was detected histologically in natural fish collected in both April and May. A higher proportion of fish collected in the Scott River to Salmon River reach were in a disease state (Cs 2 rating) when examined histologically, compared to the upstream Shasta River to Scott River reach.

Coded-wire tagged (CWT) Chinook Salmon from Iron Gate Hatchery were collected from June 4 to July 20. Due to fewer hatchery fish released and a decreased mark rate, hatchery fish were difficult to collect. For this reason, fish were not collected from the lower Klamath River. In 2020, a total of 13 CWTs were collected in the main-stem Klamath River.

In 2020, *C. shasta* infection by both QPCR and histology (prevalence of infection in natural fish, historic comparison, and annual prevalence of infection) was greater than that observed in 2019.

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Notice

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Introduction

The Klamath River watershed 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 from IGD to the Pacific Ocean (Figure 1).

The lower Klamath River basin supports 19 species of native fishes including Chinook Salmon (*Oncorhynchus tshawytscha)*, which continue to be the most abundant anadromous fish in the river (National Research 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). Since *C. shasta* and *P. minibicornis* share the same freshwater annelid host *Manayunkia occidentalis* (Atkinson et al., 2020), there is the potential for a fish to encounter actinospores from both parasites, if annelids are infected. Coinfection (concurrent multiple species infections) of *C. shasta* and *P. minibicornis* has been well documented in juvenile Chinook Salmon monitoring and sentinel studies from the Klamath River (Voss et al., 2018).

Ceratonova shasta causes enteronecrosis and contributes to mortality of juvenile salmonids 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 from 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 River (Nichols et al., 2009; True et al., 2013, Voss et al., 2018). *Parvicapsula minibicornis* accumulates in the kidney and pathology can vary from minor inflammation in lightly infected fish to glomerulonephritis and congestive necrosis of kidney tubules in heavily infected fish (True et al., 2009).

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 annelid 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).

The primary objectives of this study were to: 1) examine parasite prevalence in Klamath River juvenile Chinook Salmon during the spring out-migration period; and 2) compare parasite prevalence in 2020 to previous years.

Methods

Definitions

Definitions for report terms, such as prevalence of infection (POI), are provided in Appendix A.

Pre-Release Examination

Iron Gate Hatchery (IGH) released 989,131 brood year 2019 fall-run Chinook Salmon fingerlings on May 22, 2020. Prior to release, a fish health examination of 30 fingerlings was conducted (May 20) to determine infection levels of *C. shasta* and *P. minibicornis* by QPCR.

Collection Reaches

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

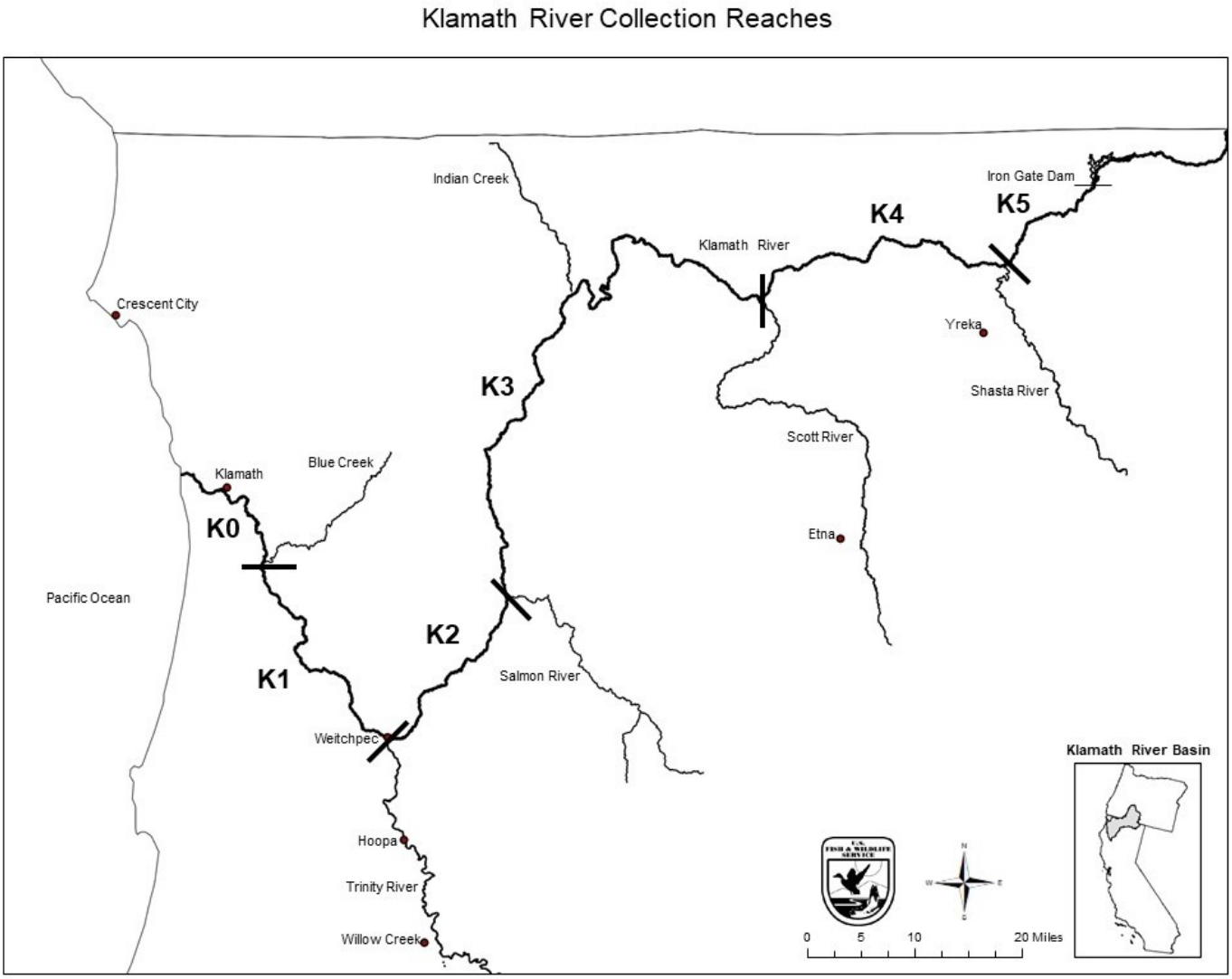


Figure 1. Klamath River watershed, major tributaries, and reaches: Iron Gate Dam to Shasta River (K5), Shasta River to Scott River (K4), Scott River to Salmon River (K3), Salmon River to Trinity River confluence (K2), Trinity River to upper Estuary (K1), and Klamath River Estuary (K0). Map provided by the USFWS Arcata Fish and Wildlife Office.

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

Collection Reach	Reach Code	River miles (Upstream – Downstream)	Primary Collector
Klamath River main stem			
Iron Gate Dam to Shasta R.[1]	K5	190-177	USFWS
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	K1	44-4	Yurok Tribe
Estuary [2]	K0	4-0	Yurok Tribe

[1] Fish have not been collected from K5 in recent years, however fish were collected in 2020 when CWT recovery was difficult.

[2] Fish were not collected from K0 in 2020.

Every year sample collection begins the fourth week in March. Sampling concludes in late August as fish migrate out of the basin and the weekly collection target can no longer be met. Sampling concluded in July 2020 for reasons described below.

In 2020, fish were collected in the main-stem Klamath River between Iron Gate Dam and Mawah Creek (a distance of ~154 river miles). When possible, rotary screw traps were used for fish collections. Beach seining was also performed to collect fish in some weeks/reaches, especially when fish were hard to locate.

Fish were collected early in the sampling season (week of March 22-June 21) in the upper reaches (K5, K4, and K3) and later in the season (week of May 31- July 19) in the lower reaches (K2 and K1) as fish were migrating downstream. The targeted weekly sample size during real-time monitoring in K4 was increased to 60 fish in 2020, compared to a 30 fish sample in 2019.

Due to fewer hatchery fish released from IGH and a decreased mark rate, CWT recovery was difficult in 2020. While trying to locate CWTs, the K5 reach was sampled in 2020. Fish have not been collected from K5 in recent years however, crews sampled this reach for four weeks starting in late May to see if marked hatchery fish were holding in the upper reach.

In 2020, very few fish were collected below the Trinity River. The Fish Health Center discussed sampling concerns with cooperators, and decided that the potential stress and associated mortality (collecting and sorting fish to search for CWTs) was not worth the sampling effort. Fish health sampling below the Trinity River confluence ceased at the end of June. Fish were not collected in the estuary in 2020. One weekly sample was collected in K1 (n=20) on the week of June 21; the sample did not include any hatchery fish.

Fish Origin

All fish collected were categorized into three groups based on their origin. A fish of natural origin is unmarked (adipose fin present) and collected prior to hatchery release.

Iron Gate Hatchery applies adipose fin clips and coded-wire tags to a portion (target is 25%) of all Chinook Salmon through their constant fractional marking program. Therefore, not all hatchery fish are fin clipped. A fish of unknown origin is unmarked (adipose fin present) and collected following

hatchery release. The term unknown origin is used as there is no way to differentiate between a natural and unmarked hatchery fish.

A fish of hatchery origin is identified by an adipose fin clip (adipose fin absent) and contains a coded-wire tag that can be extracted, read, and queried in a regional database for information such as species, brood year, release location, release date, etc.

Fish collection in the Klamath River varied by reach, with an emphasis on natural fish in the reaches below Iron Gate Dam prior to hatchery release, then unknown origin and available hatchery fish for the remainder of the spring/summer migration.

Necropsy, DNA Extraction, and Assays

Fish necropsy and DNA extraction were done according to True et al., 2013 with a few minor adjustments. First, the MagMAX Express-96 Magnetic Particle Processer was upgraded to a newer model in 2020 (ThermoFisher KingFisher Flex). The KingFisher Flex is also a magnetic bead sample processing system and operates with the same DNA extraction kits as the MagMAX. Secondly, the entire kidney and intestinal tract were not collected on larger fish later in the season. Approximately three fourths of the posterior intestine and kidney from larger fish (fork length >75mm) were removed for testing to limit sample inhibition in QPCR assays. High DNA template concentration in the QPCR reaction can inhibit amplification and result in low assay efficiency (Applied Biosystems, 2014).

Two assays were utilized to assess parasite infection; quantitative PCR and histology. Refer to Appendix B and Appendix C for detailed methodology on quantitative PCR and Appendix D for histology methods.

Results and Discussion

Pre-Release Examination of Iron Gate Hatchery Chinook Salmon

Ceratonova shasta and *P. minibicornis* were not detected in 30 juvenile Chinook Salmon reared and collected from Iron Gate Hatchery, prior to release.

Number of Fish Collected by Origin

In 2020, we tested 992 juvenile Chinook Salmon collected from the main-stem Klamath River (fish with unreadable coded-wire tags were removed from the dataset, n=2). The sample consisted of 684 natural fish, and 308 fish collected after hatchery release which included 13 CWTs.

The majority of fish collected this year were fish of natural and unknown origin (Figure 2). Natural fish accounted for 69% (684/992) of fish collected, fish of unknown origin accounted for 30% (295/992), and coded-wire tagged fish from Iron Gate Hatchery accounted for 1% (13/992).

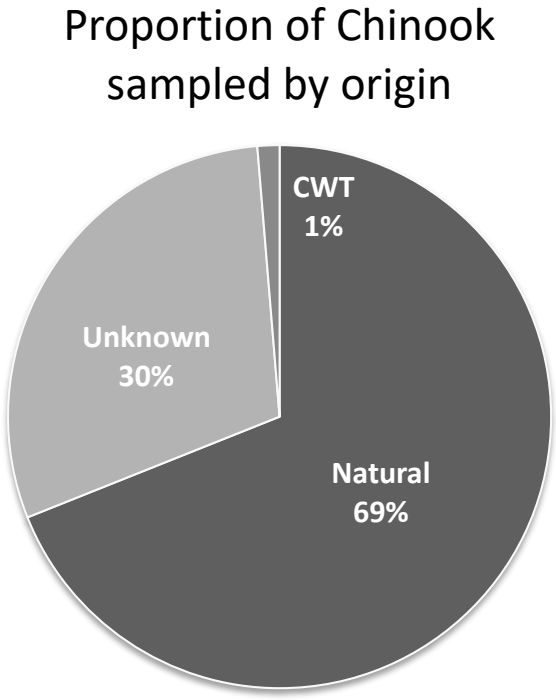


Figure 2. Proportion and origin of Chinook Salmon used for prevalence of infection analysis (N=992) in 2020.

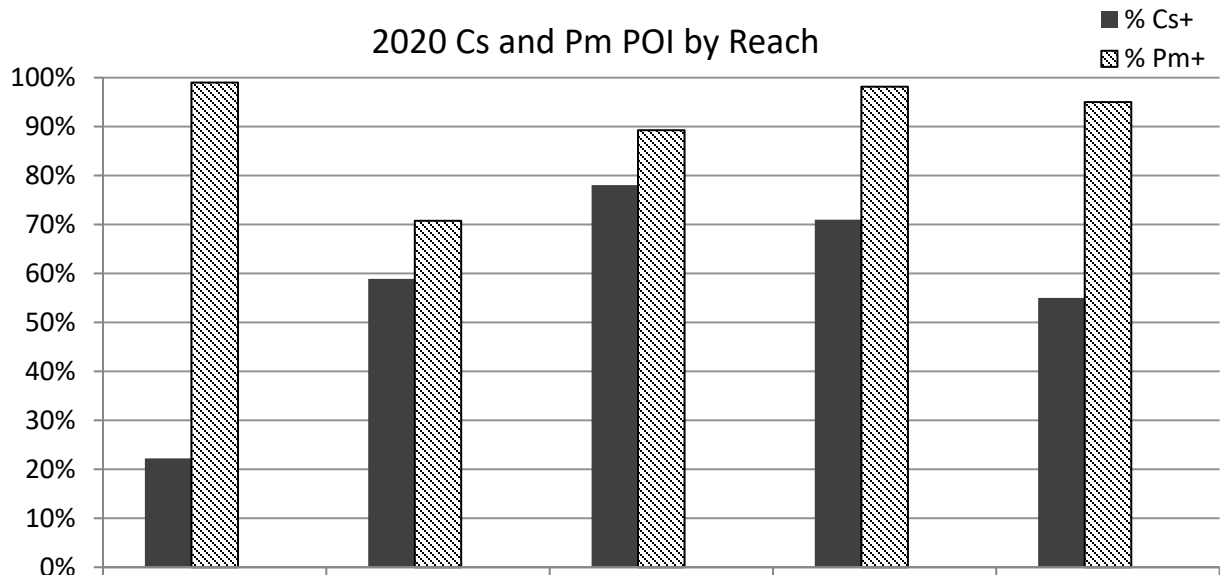
The proportion of natural and unknown origin fish increased in 2020 however, the proportion of CWTs was much smaller. In 2019, CWTs made up 28% of the fish collected, while in 2020 they accounted for 1%. It is not surprising then that the proportion of unknown fish was greater in 2020. The number of fish collected weekly and their origin is presented in Appendix E.

Hatchery fish were difficult to recover in the Klamath River in 2020 for a few reasons. First, Iron Gate Hatchery released fewer fingerlings in 2020 (989,131 fish released on May 22) due to a low number of adults returning to the hatchery in 2019 (3,628). For comparison, the hatchery released ~4.5 million juveniles in 2019. Secondly, the mark rate for CWTs was low, ~15% (Brock, 2020), compared to the target mark rate of 25%. The combination of these two factors, made it difficult to locate and collect CWTs.

Annual Prevalence of Infection by Klamath River Reach

The annual prevalence of *C. shasta* infection in all Chinook Salmon tested in 2020 by QPCR was 61% (606/992, confidence interval [ci] = 58-64%). *Ceratonova shasta* was first detected on April 8 in the Scott River to Salmon River reach (K3, Appendix E, Table E-1). Annual *C. shasta* POI was highest in the Scott River to Salmon River reach at 78%, followed by 71% in the Salmon River to Trinity River reach (K2). The *C. shasta* POI was 59% in the Shasta River to Scott River reach (K4, Figure 3).

The annual *P. minibicornis* POI in all Chinook Salmon assayed by QPCR was 82% (817/992, ci = 80-85%). *Parvicapsula minibicornis* was first detected on April 7 in the Shasta River to Scott River reach (K4, Appendix E, Table E-2). Annual prevalence was highest in the IGD to Shasta River reach (K5) at 99%, followed closely by the Salmon River to Trinity River reach (K2) at 98% (Figure 3). The lowest prevalence of 71% was observed in the Shasta to Scott reach (K4).



	K5	K4	K3	K2	K1
% Cs+	22%	59%	78%	71%	55%
% Pm+	99%	71%	89%	98%	95%
n	99	506	205	162	20

Figure 3. Prevalence of *Ceratonova shasta* (Cs+) and *Parvicapsula minibicornis* (Pm+) infection by reach in all juvenile Klamath River Chinook Salmon tested by QPCR in 2020. 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), and Trinity River to upper Estuary (K1). Sample numbers collected (n) are displayed in the table below and were the same for both pathogens.

The annual *C. shasta* POI by histology for all fish tested in 2020 was 57% (33/58, ci = 43-70%), and for *P. minibicornis* POI was 60% (35/58, ci = 47-73%). Histology results are listed in Appendix F, Table F-1 and Table F-2.

Annual *C. shasta* POI by QPCR and histology were both higher in 2020, compared to the previous year. In 2019 annual *C. shasta* POI by QPCR was 53%, and increased to 61% in 2020. *Ceratonova shasta* POI by histology increased from 20% in 2019 to 57% in 2020. The increase in POI was expected due to the environmental conditions in 2020 (lower flows and warm water temperatures).

Monitoring of waterborne stages of *C. shasta* from river water also appears to show a similar pattern of increase. In 2020, *C. shasta* was detected at a mean spore density of 158 spores/L at the Kinsman water sampling location (Oregon State University, 2020) in mid-April, compared to 7 spores/L in mid-April 2019.

Coinfection

In 2020, 0.91% (9/992, ci = 0-2%) of all fish tested by QPCR were only infected with *C. shasta*, 22% (220/992, ci = 20-25%) were only infected with *P. minibicornis*, 60% (597/992, ci = 57-63%) were coinfected, and 17% (166/992, ci = 14-19%) were uninfected.

The majority of fish were coinfected with both *C. shasta* and *P. minibicornis.* All nine fish infected only with *C. shasta* were of natural origin, and the majority of them were collected in the Scott River to Salmon River (K3) reach in mid to late April.

Over years of the monitoring program, it has been observed that most fish infected with *C. shasta* are also infected with *P. minibicornis*. In one study where the majority of fish were coinfected, fish that died of enteronecrosis and fish that survived had similar *P. minibicornis* infection levels (True et al., 2012). This suggests that *P. minibicornis* was not the driving factor of mortality in that study year of 2008. However, *P. minibicornis* has been detected at high levels by QPCR and caused clinical disease as observed by histology in 2009, 2010, 2012, and 2015 (True et al., 2016). Questions arose early in the monitoring program about whether the two parasites have a synergistic effect or if a fish infected with *P. minibicornis* has a greater chance of becoming infected with *C. shasta*. Additional studies are needed to answer these questions, but there is difficulty in obtaining or naturally exposing fish to a single myxozoan parasite.

Prevalence of Infection by Fish Origin

Naturally produced Chinook Salmon

A total of 684 natural fish were collected in the Klamath River above the Trinity River confluence for testing by QPCR. Natural fish were collected from March 24 through May 28 in Shasta River to Scott River (K4) reach, from April 8 through June 3 in the Scott River to Salmon River (K3) reach, and on June 2 in the Salmon River to Trinity River (K2) reach.

Ceratonova shasta was detected by QPCR in 64% (438/684, ci = 60-68%) of natural fish in 2020. Prevalence of infection was highest at 78% (146/186, ci = 72-84%) in the Scott River to Salmon River (K3) reach, compared to 65% (13/20, ci = 41-85%) in the Salmon River to Trinity River (K2) reach, and 58% (279/478, ci = 54-63%) in the Shasta River to Scott River (K4) reach.

The mean *C. shasta* POI for natural fish from 2009 to 2020 is 34% (1528/4489, ci = 33-35%). The POI has ranged from a low of 4% in 2012, to a high of 75-76% during the drought years of 2014-2015. *Ceratonova shasta* POI for natural fish in 2020, ranks as the third highest since 2009.

Parvicapsula minibicornis was detected in 75% (512/684, ci = 71-78%) of naturally produced Chinook Salmon by QPCR, compared to 62% in 2019. The highest *P. minibicornis* prevalence of 90% (18/20, ci = 68-99%) was detected in Salmon to Trinity (K2) reach, and the lowest prevalence of 69% (330/478, ci = 65-73%) was observed in the upper Shasta to Scott (K4) reach. Signs of clinical disease caused by *P. minibicornis* were observed during necropsy in fish of natural origin. A high percentage of fish, 98% (63/64, ci = 92-100%) with visibly swollen kidneys tested positive for the parasite by QPCR.

All juvenile Chinook Salmon examined histologically were natural origin fish collected between the Shasta River and the Salmon River (K4 and K3). A greater proportion of fish in K3 had *C. shasta* present with disease signs (histology rating of Cs 2), compared to fish collected in K4 (Appendix F, Table F-1 and Table F-2). For *P. minibicornis*, fish with disease signs (histology rating of Pm 2) were collected in May for both K4 and K3.

Real-time Monitoring

In 2020, juvenile Chinook Salmon captured from the Shasta River to Scott River (K4) reach were assayed in real-time for the first 9 weeks of the study (week of March 22 - May 17).

A comparison of real-time monitoring in previous years is presented in Appendix G. In 2020, the first *C. shasta* detection during real-time monitoring in K4 occurred in week 4, two weeks earlier than in 2019. *Ceratonova shasta* POI was 27% (16/60, ci = 16-40%) in week 4 and the POI increased weekly to peak at 98% in week 7 and week 8. *Ceratonova shasta* POI dropped to 84% during week 9, the last week of real time monitoring. This last real-time monitoring sample set was collected three days prior to hatchery release.

Comparison of POI and DNA Copy Number in Natural Fish

Weekly POI and mean DNA copy number (log scale) from natural fish collected in the upper reaches (K4 and K3) are depicted in Figure 4. The figure compares 2020 to previous years.

In the Shasta River to Scott River (K4) reach, the weekly mean DNA copy number was 1.2 logs at first detection in mid-April, peaked at 4.5 logs in early May during week 7, then dropped to 2.3 logs in late May. In the Scott River to Salmon River (K3) reach, *C. shasta* mean DNA copy number was low in early April (0.8-1.8 logs), peaking at 4.0 logs at the end of April, and decreasing to 1.7 logs in early June.

As shown in Figure 4, 2020 had the highest *C. shasta* POI and mean DNA copy number for natural fish in the last 5 years.

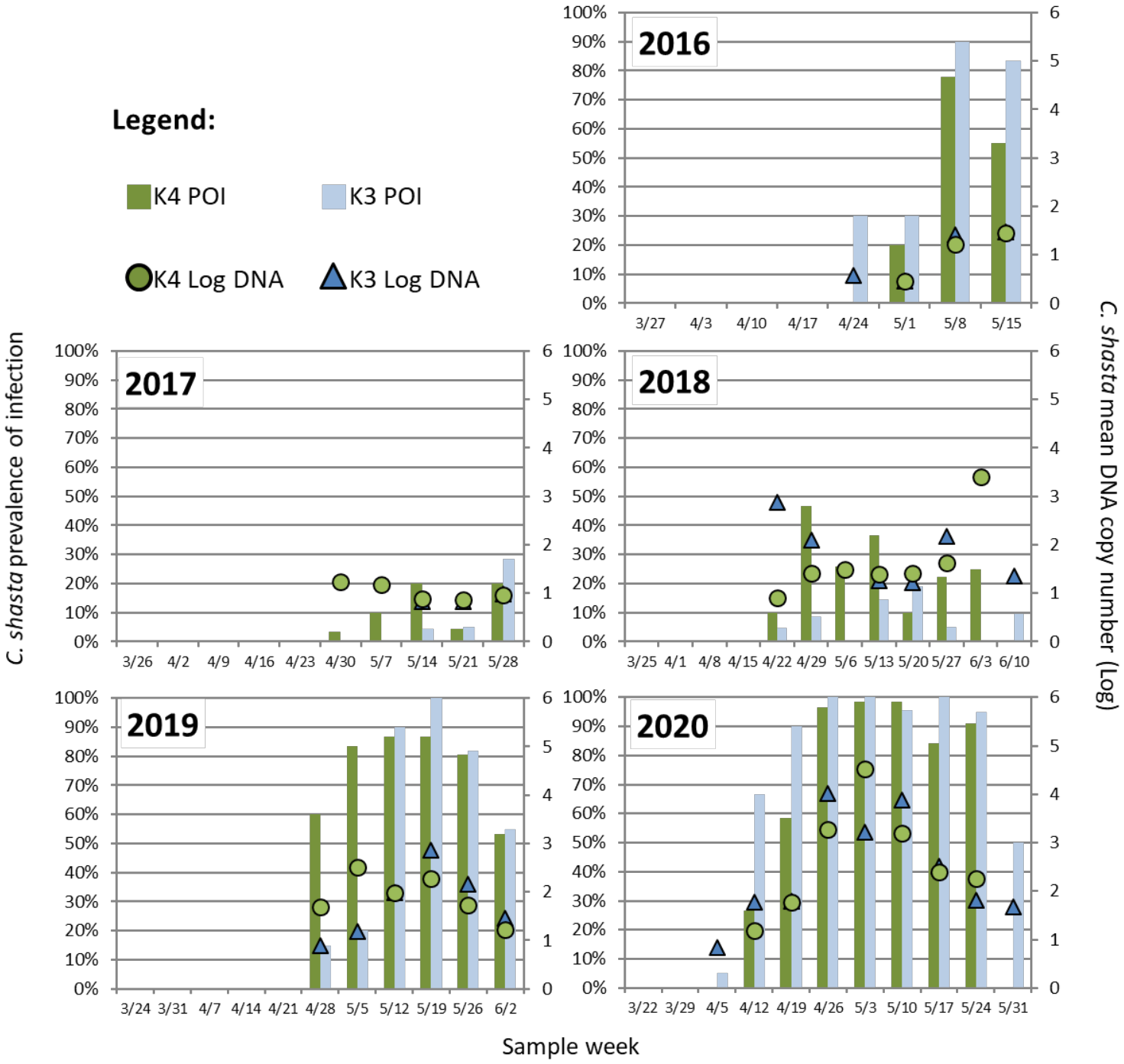


Figure 4. *Ceratonova shasta* prevalence of infection (POI) and mean DNA copy number (log) in natural juvenile Chinook Salmon captured in upper reaches: Shasta River to Scott River (K4) and Scott River to Salmon River (K3). Prevalence of infection shown in columns (Y axis) and *C. shasta* mean DNA copy number (log) shown in circles and triangles (secondary Y axis).

The "disease threshold" (2-4 logs of *C. shasta* DNA that correlates with clinical infection by histology) was first presented in our 2016 report. This threshold was removed from graphs and discussion in 2019, as we felt that data needed to be reviewed and additional field samples collected. In 2020, we collected additional fish from the Klamath River to revisit this topic and provide cooperators with a common understanding of the disease threshold used by the Fish Health Center (Appendix H). However, many of the additional fish collected in April were uninfected with *C. shasta*, therefore a larger comparative sample set is still needed to refine this relationship.

Unknown Chinook salmon

A total of 295 fish of unknown origin were collected from June 2 to July 20. *Ceratonova shasta* was detected by QPCR in 55% (162/295, ci = 49-61%) of unknown origin Chinook Salmon. Prevalence of infection peaked in late June at 94% (17/18, ci = 73-100%)

Parvicapsula minibicornis POI in fish of unknown origin was 99% (292/295, ci = 97-100%). High prevalence of infection persisted throughout the sampling season.

Iron Gate Hatchery (CWT) Chinook Salmon

Coded-wire tagged salmon originating from IGH were collected in the Klamath River from June 4 to July 20. Despite extensive sampling effort following hatchery release, only a small number of Iron Gate Hatchery CWTs were collected in 2020. Seven CWTs were recovered in the IGD to Shasta River (K5) reach, one CWT was recovered in the Shasta River to Scott River (K4) reach, and five CWTs were collected from the Salmon River to Trinity (K2) reach for a total of 13 CWTs collected. This is the smallest number of CWTs ever recovered during the Klamath River fish health monitoring project.

Ceratonova shasta was detected in 46% (6/13, ci = 19-75%) of IGH CWT screened by QPCR. Prevalence of infection for *C. shasta* was highest in K4 at 100% (one fish sample), followed by 43% in K5 and 40% in K2. *Parvicapsula minibicornis* was detected by QPCR in 100% (13/13, ci = 75-100%) of IGH CWT.

IGH CWT Weeks At Large

Due to low numbers of IGH CWT juvenile Chinook Salmon collected in 2020, Weeks at Large (WAL) analysis was not informative. The largest sample size recovered (n = 5) occurred for fish residing 1 WAL. Fish in this group had 60% *C. shasta* POI (3/5, ci = 15-95%) and a mean DNA copy number of 0.9. Again, the sample size is too small to draw any conclusions from these data.

Historical Comparison

Prevalence of *C. shasta* infection increased in both QPCR and histology in 2020, relative to previous years (Table 2.) Prevalence of *C. shasta* infection by QPCR during the peak out-migration period was 73% (433/593, ci = 69-77%) in 2020, compared to 68% in 2019, and higher than the average of 46% for the past twelve years (2009-2020).

Parvicapsula minibicornis prevalence of infection by QPCR in Chinook Salmon above the Trinity River confluence for the same time period was 99% (586/593, ci = 98-100%) compared to 96% in 2019, and also higher than the 12-year average of 85%.

Prevalence of *C. shasta* infection by histology was 60% (18/30, ci = 41-77), compared to 40% observed in 2019 (Table 2). The 12-year average of *C. shasta* infection by histology is 24%.

Table 2. Historic annual prevalence of *Ceratonova shasta* infection in all juvenile Chinook Salmon collected from the main-stem Klamath River between Iron Gate Dam and Trinity River confluence during May through July, 2009-2020. 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% [1]	(9/98)	30%	(160/526)
2013	16% [1]	(6/37)	46%	(234/508)
2014	42% [1]	(20/48)	81%	(467/576)
2015	62% [1]	(37/60)	91%	(437/482)
2016	14% [1]	(8/58)	48%	(243/504)
2017	8% [1]	(3/40)	26%	(153/600)
2018	4% [1]	(1/27)	20%	(114/570)
2019	40% [1]	(16/40)	68%	(395/581)
2020	60% [1]	(18/30)	73%	(433/593)
Mean	**24%**	**(193/795)**	**46%**	**(3090/6649)**

[1] Histology limited to two reaches in 2011 (K4 and K1); and two reaches in 2012-2020 (K4 and K3).

Environmental Conditions

Water Year 2020 was dry in Northern California (CA Department of Water Resources, 2020). A wet spring partially compensated for the dry winter months but was insufficient to restore Northern California to average conditions. Water Year 2020 also experienced a late-summer heat wave that lead to the warmest August on record for California (CA Department of Water Resources, 2020). Detailed graphs on water temperature and river flow are provided in Appendix I.

Klamath River mean water temperatures below IGD and at Seiad Valley were similar to temperature profiles from prior years, however river flows were lower in 2020. The reduced flow likely played a role in disease risk due to myxozoan parasite concentration and exposure dose.

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Photo contributions:
Cover Photo: Klamath River at Orleans, CA-NV Fish Health Center.

Author Roles

The contributions of each author have been summarized below.

- Anne Voss – Project lead and real-time sampling coordination and reporting, data management and quality control, necropsy, DNA extraction and QPCR assays, data analysis and pivot tables, and addressing reviewer comments. Primary author for 2020 annual report.
- Scott Foott – Histological sample processing and assessments for natural fish, project supervisor.
- Scott Freund – Assistance with necropsy, CWT extraction and reading.

References

Applied Biosystems. (2016). Application Note: Real-time PCR: Understanding Ct. Publication CO019879 0116. Retrieved September 2018 from Thermo Fisher Scientific: https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1503-PJ9169-CO019879-Re-brand-Real-Time-PCR-Understanding-Ct-Value-Americas-FHR.pdf

Applied Biosystems. (2014). Real-time PCR handbook. Publication CO010759 0914. Retrieved December 2018 from Thermo Fisher Scientific: https://www.thermofisher.com/content/dam/LifeTech/global/Forms/PDF/real-time-pcr-handbook.pdf

Atkinson, S. D., Bartholomew, J. L., & Rouse, G. W. (2020). The invertebrate host of salmonid fish parasites *Ceratonova shasta* and *Parvicapsula minibicornis* (Cnidaria: Myxozoa), is a novel fabriciid annelid, *Manayunkia occidentalis* sp. nov. (Sabellida: Fabriciidae). Zootaxa 4751 (2): 310–320.

Atkinson, S. D., Foott, J.S., & Bartholomew, J.L. (2014). Erection of *Ceratonova* n. gen. (Myxosporea: Ceratomyxidae) to Encompass Freshwater Species *C. gasterostea* n. sp. from Threespine Stickleback (*Gasterosteus aculeatus*) and *C. shasta* n. comb. from Salmonid Fishes. *Journal of Parasitology*, 100 (5): 640-645.

Bartholomew, J., & Foott, J. (2010). *Compilation of information relating to myxozoan disease effects to inform the Klamath Basin Restoration Agreement. Secretarial Determination Overview Report.* Retrieved Sept 25, 2013, from http://klamathrestoration.gov/sites/klamathrestoration.gov/files/Disease%20synthesis_11-1_final.bartholomew.foott.pdf

Bartholomew, J., Atkinson, S., Hallett, S., Zielinski, C., & Foott, J. (2007). Distribution and abundance of the salmonid parasite *Parvicapsula minibicornis* (Myxozoa) in the Klamath River basin (Oregon-California, U.S.A.). *Diseases of Aquatic Organisms,* 78(2), 137-146.

Bartholomew, J., Whipple, M., Stevens, D., & Fryer, J. (1997). The Life Cycle of *Ceratomyxa shasta*, a Myxosporean Parasite of Salmonids, Requires a Freshwater Polychaete as an Alternate Host. *Journal of Parasitology*, 859-868.

Bjork, S., & Bartholomew, J. (2009). Effects of *Ceratomyxa shasta* dose on a susceptible strain of rainbow trout and comparatively resistant Chinook and coho salmon. *Diseases of Aquatic Organisms,* 86, 29-37.

Brock, P. (2020). Annual Report, Iron Gate Hatchery, 2019-2020. CA Department of Fish and Wildlife, Inland Fisheries.

Bureau of Reclamation. (2020). News Releases. Reclamation to implement Klamath River flushing flow for salmon health. Klamath Basin Area Office. Retrieved December 2020 from: https://www.usbr.gov/newsroom/newsrelease/detail.cfm?RecordID=70584

CA Department of Water Resources (2020). Water Year 2020 Summary Information. Retrieved November 2020: https://water.ca.gov/-/media/DWR-Website/Web-Pages/What-We-Do/Drought-Mitigation/Files/Publications-And-Reports/Water-Year-2020-Handout_Final.pdf

Durfee, P. (1978). Prevalence and Incidence Defined. *Australian Veterinary Journal,* 54, 105-106.

Hallett, S., & Bartholomew, J. (2006). Application of real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in water samples. *Diseases of Aquatic Organisms,* 71, 109-118.

Hoffmaster, J., Sanders, J., Rohovec, J., Fyer, J., & Stevens, D. (1988). Geographic distribution of the myxosporean parasite, *Ceratomyxa shasta* Noble, 1950, in the Columbia River basin, USA. *Journal of Fish Diseases*, 97-100.

Humason, G.L. (1979) *Animal tissue techniques*. 4th ed. WH Freeman and Co., San Francisco.

Jones, S., Prosperi-Porta, G., Dawe, S., Taylor, K., & Goh, B. (2004). *Parvicapsula minibicornis* in anadromous sockeye (Oncorhynchus nerka) and coho (*Oncorhynchus kisutch*) salmon from tributaries of the Columbia River. *Journal of Parasitology*, 822-885.

National Research Council. (2004). *Endangered and Threatended Fishes in the Klamath River Basin: Causes of decline and strategies for recovery.* Washington, DC: The National Academies Press.

Nichols, K., True, K., Fogerty, R., Ratcliff, L., & Bolick, A. (2009). *FY 2008 Investigational Report: Myxosporean parasite (Ceratomyxa shasta and Parvicapsula minibicornis) incidence and severity in Klamath River basin juvenile Chinook and coho salmon, April-August 2008.* Anderson, CA.: US Fish and Wildlife Service. California-Nevada Fish Health Center.

Oregon State University. (2020). Monitoring Studies, *Ceratonova shasta* Monitoring Studies in the Klamath River. Department of Microbiology. Retrieved December 2020 from: https://microbiology.science.oregonstate.edu/content/monitoring-studies

Stocking, R., Holt, R., Foott, J., & Bartholomew, J. (2006). Spatial and Temporal Occurrence of the Salmonid Parasite *Ceratomyxa shasta* in the Oregon–California Klamath River Basin. *Journal of Aquatic Animal Health*, 194-202.

Stone, R., Foott, J., & Fogerty, R. (2008). *Comparative susceptibility to infection and disease from Ceratomyxa shasta and Parvicapsula minibicornis in Klamath River basin juvenile Chinook, coho, and steelhead populations.* Anderson, CA: US Fish and Wildlife Service. California-Nevada Fish Health Center.

True, K., Bolick, A., & Foott, J. (2013). *Myxosporean Parasite (Ceratomyxa shasta and Parvicapsula minibicornis) Annual Prevalence of Infection in Klamath River Basin Juvenile Chinook Salmon, April-August 2012.* Anderson, CA: US Fish and Wildlife Service. California-Nevada Fish Health Center.

True, K., Bolick, A., & Foott, J. (2012). *Prognosis of Ceratomyxa shasta and Parvicapsula minibicornis infections in Klamath River Coho and Trinity River Chinook Salmon.* Anderson, CA: US Fish and Wildlife Service. California-Nevada Fish Health Center.

True, K., Bolick, A., & Foott, J. (2011). *Myxosporean parasite (Ceratomyxa shasta and Parvicapsula minibicornis) annual prevalence of infection in Klamath River basin juvenile Chinook Salmon, April-August 2010.* Anderson, CA: US Fish and Wildlife Service. California-Nevada Fish Health Center.

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

True, K., Voss, A., & Foott, J. (2016). *Myxosporean Parasite (Ceratonova shasta and Parvicapsula minibicornis) Prevalence of Infection in Klamath River Basin Juvenile Chinook Salmon, April-July 2015.* Anderson, CA: US Fish and Wildlife Service. California-Nevada Fish Health Center.

USFWS. (2004, March 3). U. S. Fish and Wildlife Service. Aquatic Animal Health Policy, Series 713. In *US Fish and Wildlife Service Manual #440.*

Voss, A., True, K., & Foott, J. (2018). *Myxosporean Parasite (Ceratonova shasta and Parvicapsula minibicornis) Prevalence of Infection in Klamath River Basin Juvenile Chinook Salmon, March - August 2018.* Anderson, CA: US Fish and Wildlife Service. California-Nevada Fish Health Center.

Appendix A - Definitions and Data Analysis

Testing

Tissues from a single fish are not tested by both QPCR and histology assay methods. The majority of fish are tested by QPCR, with additional fish collected for histology.

The exception were fish collected outside the monitoring program for a pilot study in 2020; these fish were tested by both assay methods and explained in Appendix H.

Real-time Monitoring

Real-time monitoring provided timely data to fishery managers during juvenile Chinook Salmon out-migration. The goal was to provide estimates of *C. shasta* POI and DNA copy number by QPCR to managers on a weekly basis. Histology samples were not reported in real-time. Real-time monitoring occurred in the Shasta River to Scott River reach (K4) prior to hatchery release. The Kinsman rotary screw trap was the primary collection site for real-time monitoring.

The Kinsman rotary screw trap was deployed at the beginning of the sample week for fish collection. Field crews collected fish from the trap, euthanized, bagged fish with collection tag, 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. The Fish Health Center required that fish were transported to the laboratory no later than Wednesday afternoon in order 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, 2004) for *C. shasta and P. minibicornis* are reported with 95% confidence intervals (denoted ci) for each sample reach. 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.

Comparison of POI and DNA Copy Number in Natural Fish

In order 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, prevalence of infection 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 heavily infected, lightly infected, etc. It is informative to also consider DNA copy number (how much parasite DNA is in the fish tissue) in order to get a more complete picture of infection.

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 0 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.

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 period of May 1 to July 31 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 6FAMCGA 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 replicate. Reactions were assayed using a 7300 Sequence Detection 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 (SDS software 7300 SDS v 1.4, Applied Biosystems). The standard curve was used to evaluate PCR amplification efficiency (slope of the standard curve, efficiency was 94%), fit to the curve (R^2 value = 0.997), and the y-intercept (39.1) 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. One *C. shasta* assay plate was retested in 2020.

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).

Appendix C – Methods for *Parvicapsula minibicornis* Quantitative PCR Assay

A *Parvicapsula minibicornis* quantitative PCR assay targeting the 18S ribosomal subunit gene was used to assay DNA extracted from fish tissues. Forward primer (Pm-176F: CAA GGA GCCCGG AGC ATT), reverse primer (Pm-267R: TTA CAT TCA ACT AAATCA CAC ACT GTT ACA), and minor groove binding probe (Pm-210T: CCA ACA GCATAC ATT GGT) sequences were used from True et al., 2009.

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 replicate. Reactions were assayed using a 7300 Sequence Detection 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 *P. minibicornis* reference standard curve was obtained by using plasmid DNA containing the 18S ribosomal DNA target sequence. Specifically, 1 ng of DNA, corresponding to 2.31×10^8 copies of *P. minibicornis* DNA was serially diluted over eight orders of magnitude in molecular grade water. The standard curve was used to evaluate PCR amplification efficiency (slope of the standard curve, efficiency was 100.2%), fit to the curve (R^2 value = 0.995) and the y-intercept (41.4, C_T value for a single copy of parasite DNA).

Each assay was evaluated for expected CT 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. Two *P. minibicornis* assay plates were retested in 2020.

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).

Appendix D - Methods for Histology Assay

Histological assays were done to assess clinical disease (disease severity that results in tissue damage). In 2020, fish for histology were collected the week of April 12 and May 10 in the Shasta River to Scott River (K4) reach and biweekly between the week of April 12 and May 24 in the Scott River to Salmon River (K3) reach.

Fish tested by histology were euthanized and quickly placed in Davidson's fixative. Fish were held in fixative for 24-48 hours. The fixative was replaced with 70% ethanol for storage until the gross examination and histological processing were performed. Each histological cassette contained intestine (target tissue for *C. shasta*), kidney (target tissue for *P. minibicornis*), and 1 to 2 gill filaments. Specimens were processed for 5µm paraffin sections and stained with hematoxylin and eosin (Humason, 1979). Tissues from each fish were placed on one slide and identified by a unique number code. Each slide was examined at 40X to 400X magnification.

Histological assays were assigned a rating of clinical disease based on the presence of multifocal lesions associated with parasite infection; see Table D-1 below. Any parasites or abnormalities in gill tissue were also noted.

Table D-1. Histological disease rating definitions for *C. shasta* and *P. minibicornis*. Definitions for abbreviations listed in the histological result tables.

Intestinal tract	
Histological Disease Rating, Cs 0	Absence of parasite in tissue
Histological Disease Rating, Cs 1	Presence of parasite in tissue, but with minimal inflammatory changes (parasite present without disease signs)
Histological Disease Rating, Cs 2	Presence of parasite in tissue with hyperplasia, lamina propria, necrotic epithelium and/or necrotic muscularis (parasite present with disease signs)
Kidney	
Histological Disease Rating, Pm 0	Absence of parasite in tissue
Histological Disease Rating, Pm 1	Presence of parasite in tissue, but with minimal inflammatory changes (parasite present without disease signs)
Histological Disease Rating, Pm 2	Presence of parasite in tissue with interstitial hyperplasia, necrotic interstitium or tubule, glomerulonephritis, and/or protein casts within the glomeruli or tubules (parasite present with disease signs)
Gill	
Metacercaria	Immature trematode stage
Multif. Hyperplasia	Multifocal hyperplastic regions on lamellae

Appendix E – QPCR Results

Table E-1. *Ceratonova shasta* infection by QPCR in juvenile Chinook Salmon sampled from five 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	Iron Gate Dam 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)
1	22-Mar		0% (0/30)[A]			
2	29-Mar		0% (0/30)[A]			
3	5-Apr		0% (0/58)[A]	5% (1/20)[A]		
4	12-Apr		27% (16/60)[A]	67% (14/21)[A]		
5	19-Apr		58% (35/60)[A]	90% (18/20)[A]		
6	26-Apr		97% (58/60)[A]	100% (20/20)[A]		
7	3-May		98% (59/60)[A]	100% (21/21)[A]		
8	10-May		98% (59/60)[A]	95% (21/22)[A]		
9	17-May		84% (32/38)[A]	100% (23/23)[A]		
10	24-May		91% (20/22)[A]	95% (18/19)[A]		
11	31-May	33% (19/58)[C]	100% (5/5)[B]	50% (10/20)[A]	65% (13/20)[A]	
12	7-Jun	9% (1/11)[C]	67% (8/12)[C]	74% (14/19)[B]	95% (19/20)[B]	
13	14-Jun	7% (1/14)[C]	44% (4/9)[B]		55% (11/20)[C]	
14	21-Jun	6% (1/16)[B]	100% (2/2)[B]		50% (10/20)[B]	55% (11/20)[B]
15	28-Jun				89% (17/19)[C]	
16	5-Jul				75% (15/20)[B]	
17	12-Jul				59% (13/22)[C]	
18	19-Jul				81% (17/21)[C]	
		K5 Total **22% (22/99)**	K4 Total **59% (298/506)**	K3 Total **78% (160/205)**	K2 Total **71% (115/162)**	K1 Total **55% (11/20)**

[A] Fish of natural origin.

[B] Fish of unknown origin.

[C] Weekly sample contains both fish of unknown and hatchery (CWT) origin.

Table E-2. *Parvicapsula minibicornis* infection by QPCR in juvenile Chinook Salmon sampled from five 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	Iron Gate Dam 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)
1	22-Mar		0% (0/30)[A]			
2	29-Mar		0% (0/30)[A]			
3	5-Apr		3% (2/58)[A]	60% (12/20)[A]		
4	12-Apr		77% (46/60)[A]	48% (10/21)[A]		
5	19-Apr		77% (46/60)[A]	90% (18/20)[A]		
6	26-Apr		97% (58/60)[A]	100% (20/20)[A]		
7	3-May		98% (59/60)[A]	100% (21/21)[A]		
8	10-May		100% (60/60)[A]	100% (22/22)[A]		
9	17-May		97% (37/38)[A]	100% (23/23)[A]		
10	24-May		100% (22/22)[A]	95% (18/19)[A]		
11	31-May	98% (57/58)[C]	100% (5/5)[B]	100% (20/20)[A]	90% (18/20)[A]	
12	7-Jun	100% (11/11)[C]	100% (12/12)[C]	100% (19/19)[B]	95% (19/20)[B]	
13	14-Jun	100% (14/14)[C]	100% (9/9)[B]		100% (20/20)[C]	
14	21-Jun	100% (16/16)[B]	100% (2/2)[B]		100% (20/20)[B]	95% (19/20)[B]
15	28-Jun				100% (19/19)[C]	
16	5-Jul				100% (20/20)[B]	
17	12-Jul				100% (22/22)[C]	
18	19-Jul				100% (21/21)[C]	
		K5 Total **99% (98/99)**	K4 Total **71% (358/506)**	K3 Total **89% (183/205)**	K2 Total **98% (159/162)**	K1 Total **95% (19/20)**

[A] Fish of natural origin.

[B] Fish of unknown origin.

[C] Weekly sample contains both fish of unknown and hatchery (CWT) origin.

Appendix F – Histological Summary

Table F-1. Parasite prevalence of infection [% (number positive/number tested)], disease rating for kidney and intestine (number of fish in each rating category), 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 Sunday of given week.

Collection week	April 12	May 10	2020 POI
Intestinal tract			
Cs POI	25% (2/8)	70% (7/10)	50% (9/18)
Histological Disease Rating, Cs 0	6	3	9
Histological Disease Rating, Cs 1	2	3	5
Histological Disease Rating, Cs 2	0	4	4
Kidney			
Pm POI	0% (0/8)	100% (10/10)	56% (10/18)
Histological Disease Rating, Pm 0	8	0	8
Histological Disease Rating, Pm 1	0	4	4
Histological Disease Rating, Pm 2	0	6	6
Gill			
Metacercaria	13% (1/8)	70% (7/10)	44% (8/18)
Multif. Hyperplasia	0% (0/8)	40% (4/10)	22% (4/18)

Table F-2. Parasite prevalence of infection [number positive / number tested (%)], disease rating for kidney and intestine (number of fish in each rating category), 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 Sunday of given week.

Collection week	April 12	April 26	May 10	May 24	2020 POI
Intestinal tract					
Cs POI	30% (3/10)	100% (10/10)	70% (7/10)	40% (4/10)	60% (24/40)
Histological Disease Rating, Cs 0	7	0	3	6	16
Histological Disease Rating, Cs 1	3	4	1	3	11
Histological Disease Rating, Cs 2	0	6	6	1	13
Kidney					
Pm POI	0% (0/10)	80% (8/10)	100% (10/10)	70% (7/10)	63% (25/40)
Histological Disease Rating, Pm 0	10	2	0	3	15
Histological Disease Rating, Pm 1	0	8	1	1	10
Histological Disease Rating, Pm 2	0	0	9	6	15
Gill					
Metacercaria	20% (2/10)	50% (5/10)	90% (9/10)	80% (8/10)	60% (24/40)
Multif. Hyperplasia	10% (1/10)	30% (3/10)	60% (6/10)	90% (9/10)	48% (19/40)

Appendix G – Real-time Monitoring

Table G-1. *Ceratonova shasta* POI in natural juvenile Chinook Salmon collected from the Shasta River to Scott River reach (K4) in 2015-2020 during real-time monitoring.

Year	Sample Week	Capture Date	Number *C. shasta* positive	Number of Fish Tested	Cs POI
2015	1	4/02/2015	0	20	0%
	2	4/09/2015	4	20	20%
	3	4/16/2015	7	20	35%
	4	4/22/2015	18	20	90%
	5	4/30/2015	20	20	100%
	6	5/07/2015	20	20	100%
	7	5/14/2015	18	20	90%
	8	5/21/2015	19	20	95%
2016	1	3/31/2016	0	20	0%
	2	4/07/2016	0	20	0%
	3	4/14/2016	0	19	0%
	4	4/21/2016	0	21	0%
	5	4/28/2016	0	20	0%
	6	5/05/2016	4	20	20%
	7	5/12/2016	14	18	78%
	8	5/19/2016	11	20	55%
2017	1	3/28/2017	0	30	0%
	2	4/03/2017	0	30	0%
	3	4/10/2017	0	30	0%
	4	4/17/2017	0	30	0%
	5	4/24/2017	0	30	0%
	6	5/02/2017	1	30	3%
	7	5/08/2017	3	30	10%
	8	5/15/2017	6	30	20%
	9	5/22/2017	1	23	4%
	10	5/30/2017	6	30	20%

2018	1	3/26/2018	0	30	0%
	2	4/02/2018	0	30	0%
	3	4/09/2018	0	30	0%
	4	4/16/2018	0	30	0%
	5	4/23/2018	3	30	10%
	6	4/30/2018	14	30	47%
	7	5/07/2018	8	31	26%
	8	5/14/2018	11	30	37%
	9	5/21/2018	3	30	10%
	10	5/29-6/01/2018	2	9	22%
	11	6/04-6/06/2018	1	4	25%
2019	1	3/26/19	0	30	0%
	2	4/02/19	0	30	0%
	3	4/09/19	0	30	0%
	4	4/16/19	0	30	0%
	5	4/23/19	0	30	0%
	6	4/30-5/02/19	18	30	60%
	7	5/07/19	25	30	83%
	8	5/13/19	26	30	87%
	9	5/20/19	26	30	87%
2020	1	3/24-3/25/2020	0	30	0%
	2	4/01/2020	0	30	0%
	3	4/07/2020	0	58	0%
	4	4/14/2020	16	60	27%
	5	4/21/2020	35	60	58%
	6	4/28/2020	58	60	97%
	7	5/05/2020	59	60	98%
	8	5/12/2020	59	60	98%
	9	5/19/2020	32	38	84%

Appendix H – Correlation of Histological Disease Score with DNA Copy Number

In April 2020, a pilot study was conducted to determine the relationship between copy number and histological rating of tissue for both *Ceratonova shasta* infection of intestine and *Parvicapsula minibicornis* infection of kidney.

Fifteen Chinook Salmon fry each were collected at the USFWS Arcata FWO rotary screw traps at I-5 (April 22, 2020) and Kinsman (April 28, 2020). These samples were outside of the standard collections for fish health monitoring and required careful dissection of the intestine and kidney to obtain comparable samples for both QPCR and histological analysis. The distal and anterior regions of both organs divided between the 2 assays. Assay methodology was the same as outlined in this report (DNA copy number derived from QPCR analysis and histology disease rating 0, 1, or 2).

Ceratonova shasta was not observed in the intestine of fish collected at the I-5 screw trap by histology; only two of 15 samples were positive for *C. shasta* by QPCR with low copy numbers. Similarly, *P. minibicornis* trophozoites were not observed in the kidney by histology, and seven of 15 samples were positive for *P. minibicornis* by QPCR with low copy numbers.

At the Kinsman trap site a *C. shasta* copy number of 1000 or more was detected in nine fish, with six fish rated as diseased (Cs 2 rating) by histology (Figure H-1). The samples with *C. shasta* copy number of > 2000 were all rated as Cs 2 by histology. No correlation with kidney inflammation (disease) and P. *minibicornis* copy number was observed in the sample set. Further comparative sampling should be done to refine the "diseased" copy number threshold.

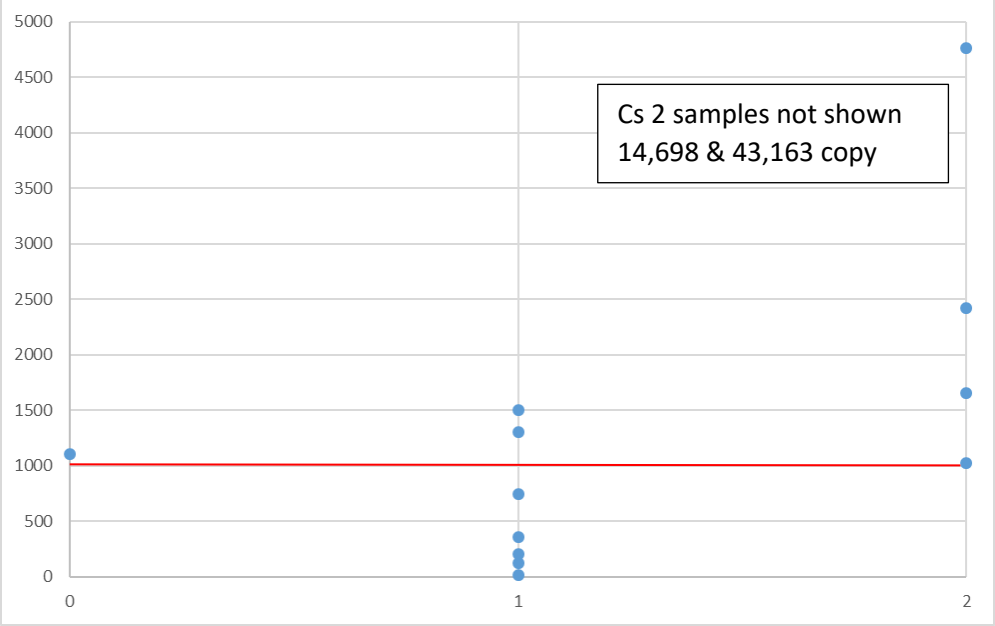

Figure H-1. *C. shasta* copy number (y-axis) to histology rating (x-axis). DNA copy number of 1000 is highlighted in red. Two Cs 2 fish not shown for graphic scale issues.

For more detailed information, refer to "Correlation of histological disease score with *C. shasta* and *P. minibicornis* copy number in Chinook Fry. Pilot study April 2020" located on the Fish Health Center website. Available at https://www.fws.gov/canvfhc/CANVReports.html

Appendix I – Environmental Conditions

River Water Temperature

Water temperatures for the spring and summer of 2020 were similar to 2019. In 2020, water temperatures below Iron Gate Dam started out low in early March (approximately 6ºC) and climbed steadily to 15.3ºC on April 30. Temperatures increased gradually to 20ºC by late July, reaching a peak of 21.2 ºC on August 29, and decreasing afterwards (Figure 1).

In previous study years, we typically observed mean daily water temperatures of approximately 18ºC, and often as high as 22ºC below Iron Gate Dam, during the peak juvenile migration period of May through July. In 2020, the mean daily water temperature was 17.2ºC (ranging from 13.9 - 20.2ºC) during this period.

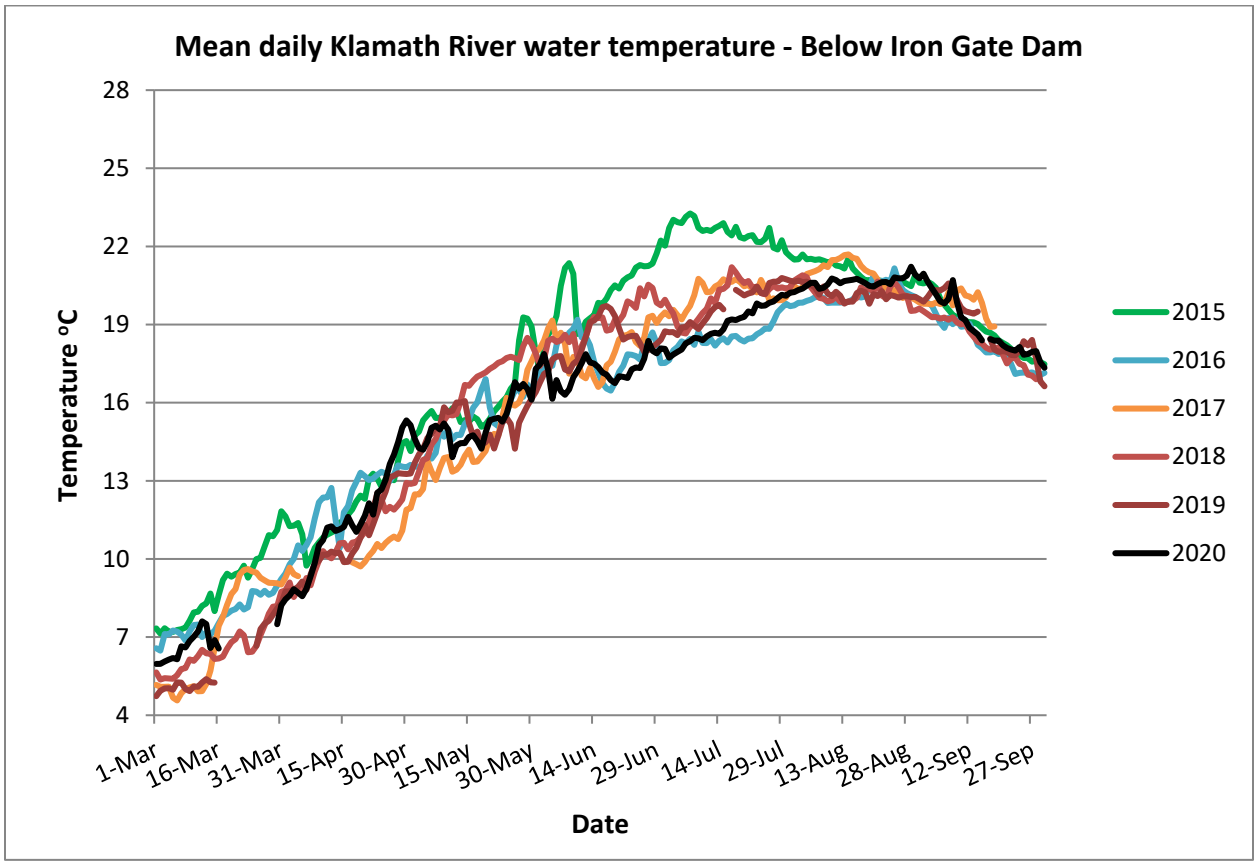

Figure I-1. Mean daily Klamath River water temperature below Iron Gate Dam for 2015-2020. Temperature data for 2015 were acquired from USFWS Arcata Fish and Wildlife Office. Temperature data for 2016-2020 were acquired from the Karuk Tribe.

Another temperature gage is located in the Scott River to Salmon River (K3) reach, near Seiad Valley. Unlike the water temperatures below Iron Gate Dam this gage has less influence from the dam and the water temperatures are more variable. When data were accessed (November 2020) the Seiad Valley temperature gage was missing data for the end of May and all of June. Water temperatures at Seiad Valley were approximately 8.5ºC in March and climbed steadily to 17.3ºC on May 10 (Figure 2). Temperatures increased through July to a peak at Seiad Valley at 25.5ºC on July 20.

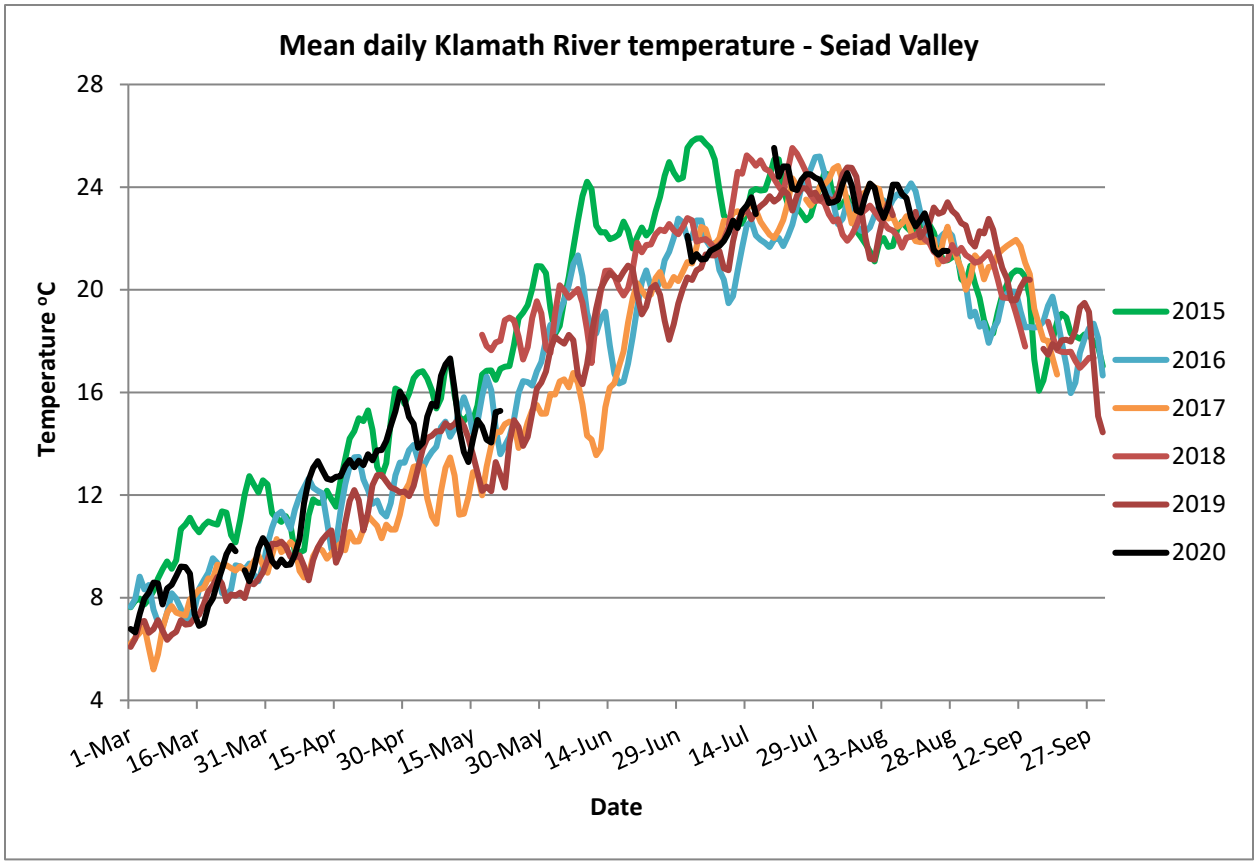

Figure I-2. Mean daily Klamath River temperature from March through September 2015-2020 at Seiad Valley. Data from 2015 were provided by the USFWS Arcata Fish and Wildlife Office. Temperature data for 2016-2020 were acquired from the Karuk Tribe.

In previous study years, we typically observed mean daily water temperatures of approximately 18ºC, and often as high as 24ºC at Seiad Valley, during the peak juvenile migration period of May through July. That trend held true in 2020 as the mean daily water temperature during peak juvenile migration was 19.6ºC (ranging from 13.3 - 25.5ºC).

River Flows

Klamath River flow below Iron Gate Dam was ~1,000 cfs early in the year and then increased to ~1,400 cfs in early April (Figure I-3). The Bureau of Reclamation implemented a surface flushing flow starting on April 22, which peaked below Iron Gate Dam at 6,440 cfs on April 23 (Bureau of Reclamation, 2020). Flows decreased to ~1,200 in late May. The minimum flow observed during the sampling season was 883 cfs on August 8.

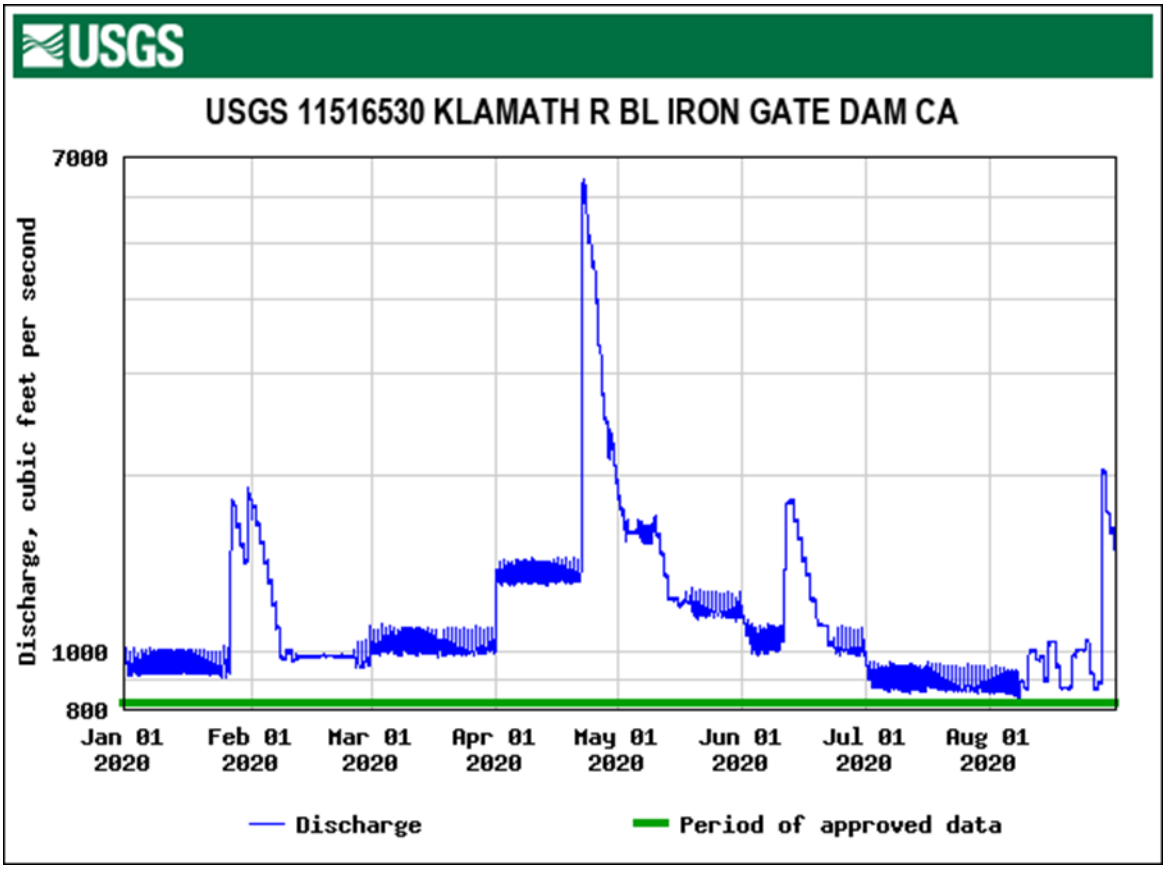

Figure I-3. Daily discharge (cfs) below Iron Gate Dam from January 2020 through August 2020. Data collected from USGS gaging station 11516530 at waterdata.usgs.gov.

As is the case with water temperature, the hydrograph from Seiad Valley shows greater variability over the summer, compared to flows below IGD. The peak flow at Seiad Valley was associated with the surface flushing flow event on April 24 at 7,270 cfs (Figure I-4). Flows decreased gradually the remainder of the summer. The minimum flow observed during the sampling season was 949 cfs on August 8.

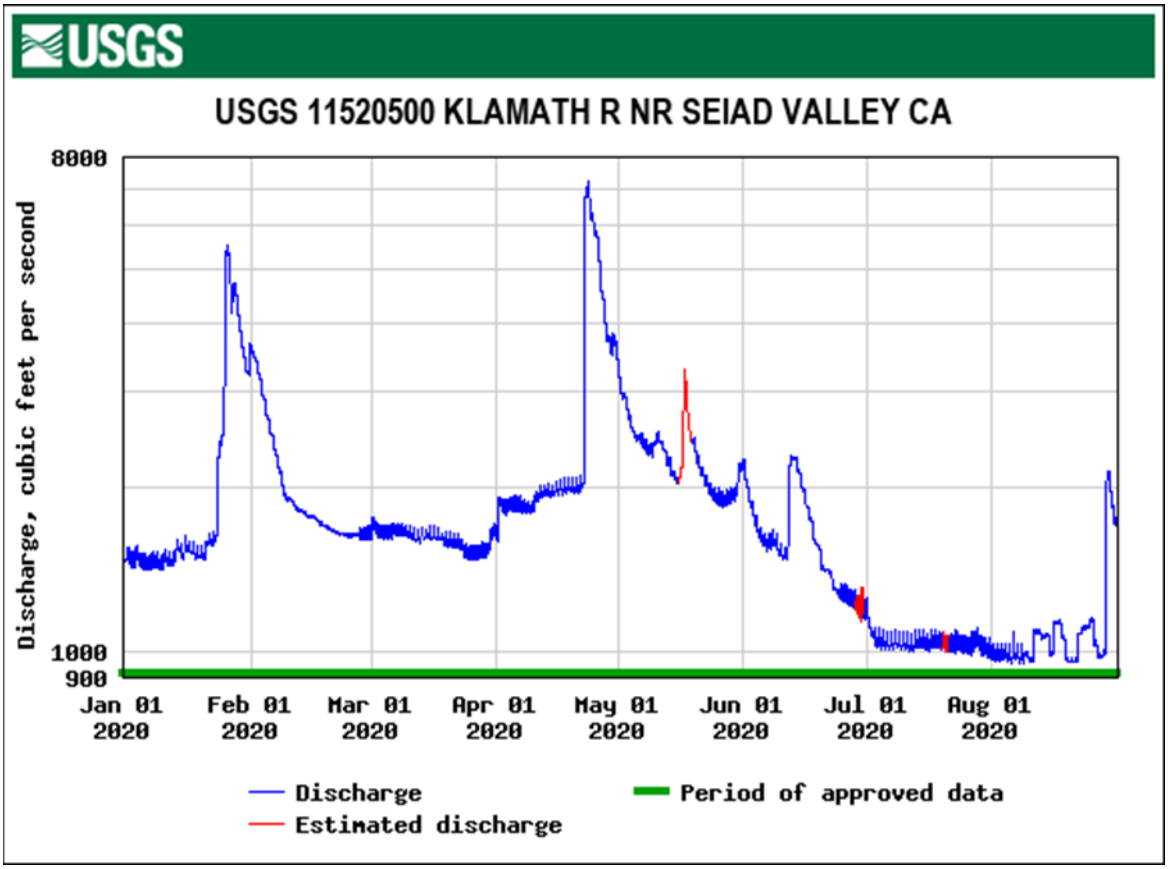

Figure I-4. Daily discharge (cfs) near Seiad Valley from January 2020 through August 2020. Data collected from USGS gaging station 11520500 at waterdata.usgs.gov.

Daily river discharge below Iron Gate Dam for the last twelve years is shown in Figure I-5. In 2009 and 2010 flows did not reach above 2,000 cfs. In 2011, two peak spring flows exceeded 5,000 cfs, the first of which was a manipulated pulse flow released from Iron Gate Dam. In 2012, spring flows were close to 4,000 cfs. Both 2014 and 2015 were ranked as extreme drought years. In 2017 flows remained above 2,000 cfs for most of the spring and summer, as 2017 was a wet year. Flows in 2018 were low, with most of the year at less than 2,000 cfs except for the peaks when U.S. Bureau of Reclamation released water.

Klamath River flows in 2020 were lower compared to the wet 2019 Water Year. In 2020, a greater portion of the year had flows recorded below 2,000 cfs.

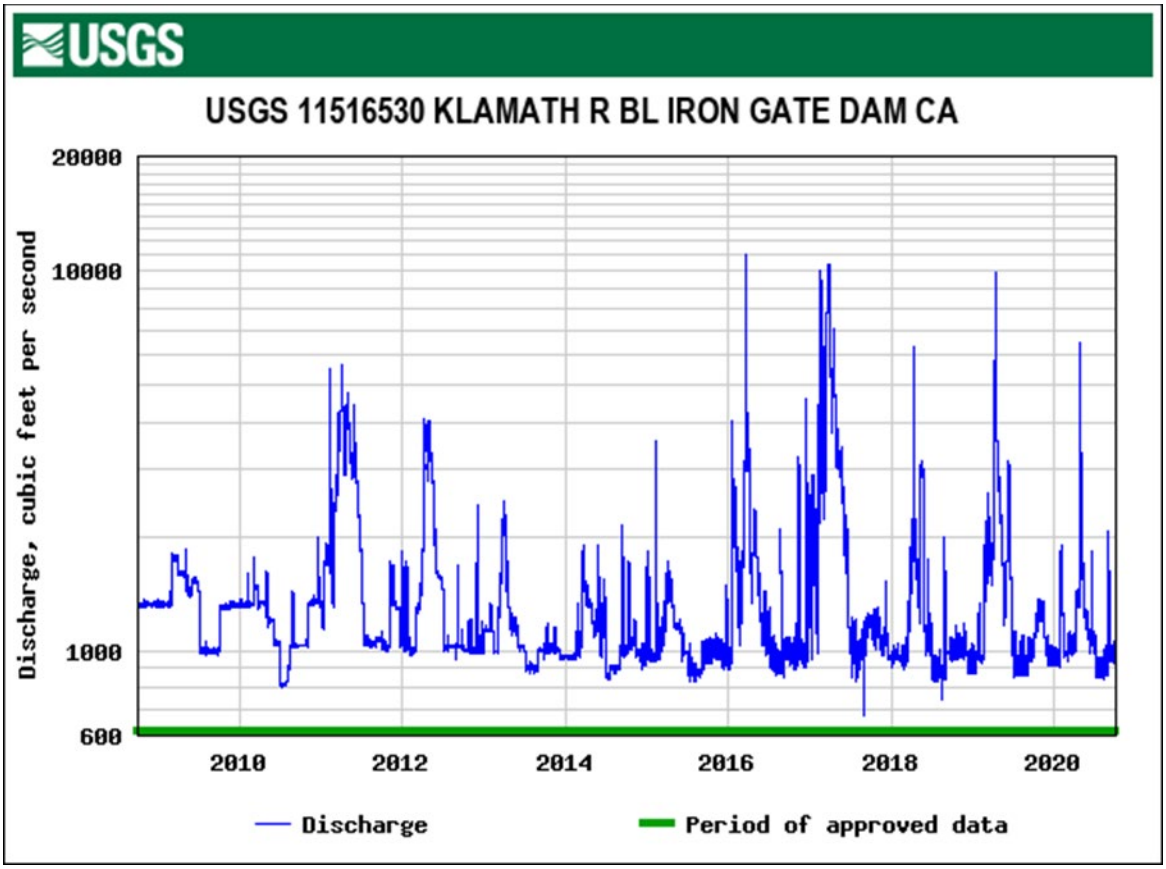

Figure I-5. Daily discharge (cfs) below Iron Gate Dam from October 2008 through September 2020. Data acquired from USGS waterdata.usgs.gov

Appendix J - Reviewer comments

Significant comments provided by reviewers of this report are included below. The author's response is given below each comment.

Reviewer #1

General comments: The reviewer had minor grammatical corrections and suggestions about sentence structure throughout the text.

Response: Grammar and sentence suggestions were corrected at the discretion of the authors.

Pg. 9 – Naturally produced Chinook Salmon: The reviewer requested for more detail about the swollen kidneys observed during necropsy. What proportion had swollen kidneys? Were these fish tested by QPCR or histology?

Response: The authors were trying to convey that clinical signs of disease from *P. minibicornis* infection were observed grossly during necropsy. The sentence has been edited to communicate that a very high percentage (98%) of fish with swollen kidneys observed during necropsy tested positive by QPCR.

Pg. 9 – POI and DNA Copy Number: The reviewer stated that discussing DNA copy number in log scale is awkward and suggested changing to a linear scale.

Response: Concentrations in QPCR are often transformed to log scale given the wide range in values. It might be somewhat awkward to read however the values also accompany the following graph, therefore we are going to discuss DNA copy number in logarithmic scale.

Pg. 20 and Pg. 21 – Appendix B and C, Methods for QPCR assay: The reviewer asked what type of replication (duplicate or triplicate) is used on the extracted DNA samples during QPCR.

Response: Language was edited to inform the reader that we assay one well per unknown sample, therefore it is not replicated.

Pg. 25 and 26 – Appendix F, Histological Summary: The reviewer suggested that a column be added to Appendix F to summarize the total values.

Response: There are totals columns already included in Appendix F. That was unclear, therefore the column headings have been renamed.

Reviewer #2

General comments: The reviewer had minor grammatical corrections throughout the text.

Response: Grammar and punctuation were corrected at the discretion of the authors.

General comments: Water temperature and flow impact disease severity, and Reclamation completed a flushing flow in 2020 to offset impacts. A section describing the benefits or impacts of these factors would be useful to readers, especially in relation to POI.

Response: A more robust analysis of environmental conditions relative to POI/DNA copies/histology results is beyond the scope of this technical report.

General comments: It is unclear to the reviewer if a fish is tested by both assay methods (QPCR and histology).

Response: Language added to Appendix A to clarify that fish are not tested by both assay methods. The exception, in 2020, were pilot study fish collected outside of the monitoring project and described in Appendix H.

Pg. 3 and Pg. 4 – Introduction and Methods: Reviewer commented that both "lower river" and "middle river" are used to describe the river below Iron Gate Dam.

Response: Language in the report was corrected to describe the "lower basin" as the Klamath River below Iron Gate Dam. The Klamath River below the confluence with the Trinity River is referred to in the report as the "lower river."

Pg. 5 – Methods, Collection Reaches: Reviewer commented when discussing the reduced mark rate and difficulty in collecting CWTs in 2020, it would be helpful to know the hatchery mark rate from previous years. It would be helpful to clarify that not all hatchery fish are clipped.

Response: Fish origin definitions were expanded on page five to explain to the reader that not all hatchery fish are fin clipped. Mark rate details were also added to the results and discussion section on page seven. The constant fractional marking program target at IGH is 25%.

Pg. 5 – Methods, Collection Reaches: Reviewer asked how the sampling season is determined.

Response: Language added to page five to explain to the reader that the start of the sampling season occurs every year in late March. The conclusion of the sampling season is fluid and depends on the availability of fish as they are out-migrating. When weekly sample collection targets can no longer be reached, the season is ended.

Pg. 6 – Necropsy, DNA Extraction, and Assays: Reviewer commented that it is not clear why the entire kidney and intestinal tract are not used for testing larger fish.

Response: Clarification added on page six to explain that the entire organ is not used for testing larger fish because too much tissue (and therefore too much DNA) can lead to the sample not amplifying correctly during QPCR.

Pg. 6 – Number of Fish Collected by Origin: Reviewer suggested adding a table to accompany the pie graph. This would allow the reader to see the actual numbers separated by reach. For example, the table could show where the majority of unknown fish were captured. Currently, that information cannot be obtained from the pie graph.

Response: Footnotes were added to Appendix E. Footnotes inform the reader of fish origin for weekly sample collections.

Pg. 10 – Real-time Monitoring: Reviewer suggested providing information on what "real-time" means and details of that process.

Response: Details about real-time monitoring was added to Appendix A.

Pg. 11 – Iron Gate Hatchery (CWT Chinook Salmon: Reviewer commented that more details are needed about the Iron Gate Hatchery release in order for the reader to understand the sampling effort and difficulty collecting CWTs. It would be useful to know the number of fish released each year, the release dates, fish sizes, and any changes to release schedules due to disease severity, water temperature, or flows.

Response: Details of the Iron Gate Hatchery release were added to page four. The hatchery released 989,131 brood year 2019 fall-run Chinook Salmon fingerlings all on a single day (May 22, 2020). Language was also added to the report on page seven to give context to the reader about the release size the previous year.

Pg. 13 – Historical Comparison: Reviewer suggested adding an additional column in Table 2 to include mean DNA copy number that accompanies the QPCR results.

Response: Authors will consider adding DNA copy number to table or revising historic comparison in future reports. Prevalence of infection by QPCR has been the metric for historical comparison since 2009, and for that reason DNA copy number was not addressed when discussing historical comparison.

Pg. 20 – Appendix B, Methods for *Ceratonova shasta* Quantitative PCR Assay: Reviewer asked if there is a reference where the reader can find the QPCR standard curves.

Response: The QPCR standard curves are produced annually based on material of known copy number, therefore there is no literature to reference for the standard curves. Our standards are run in parallel with unknown samples, therefore every assay plate has its own standard curve.

Pg. 22 – Appendix D - Methods for Histology Assay: Reviewer suggests explaining why the kidney, intestine, and gill are used for histological examination. It could be unclear to the reader which parasite occurs in tissues.

Response: Details were added to the text to explain the target tissue for each parasite and how the tissues are examined by histology.

Pg. 23 – Appendix E and Appendix F: Reviewer recommends that tables in Appendix E and Appendix F are formatted the same.

Response: Agreed; table formatting has been updated.

Reviewer #3

General comments: The reviewer suggested that the term “main stem” used throughout the report should be changed to main-stem, with a hyphen.

Response: The American Fisheries Society suggests that main stem be written as two words when used as a noun, and written as a compound word when used as an adjective. Therefore, we agree with the reviewer and the term has been changed to main-stem.

General comments: The reviewer had minor grammar and punctuation comments throughout the report.

Response: Grammar and punctuation were corrected at the discretion of the authors.