U.S. Fish & Wildlife Service

California-Nevada Fish Health Center
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

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SUMMARY

Juvenile Klamath River Chinook (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*) experience high incidence of infection with the myxosporean parasites *Ceratomyxa shasta* and *Parvicapsula minibicornis* during the spring and summer outmigration period. Klamath River Chinook and coho were assayed by histology or quantitative real-time polymerase chain reaction (QPCR) to determine parasite infection rates from April to August, 2008. The incidence of *C. shasta* assayed by histology in Chinook salmon was high, with levels similar to those observed during Klamath River parasite monitoring studies 2004 and 2005. The incidence of *P. minibicornis* by histology in Chinook salmon was also high, and near the record levels seen in 2005. The QPCR assay results from marked Iron Gate Hatchery (IGH) and Trinity River Hatchery (TRH) Chinook salmon, suggests that Klamath River reaches above the Trinity River confluence were more infectious when compared to Klamath River reaches below the Trinity River confluence, particularly for *C. shasta*. In marked IGH Chinook salmon screened by QPCR, *C. shasta* was detected in 27%, *P. minibicornis* incidence was detected in 72%, and weekly infection prevalence peaked by the third week following hatchery release. The incidence of *C. shasta* infection remained low in marked TRH Chinook salmon sampled in the Klamath River and was similar to the 1% incidence observed in Chinook salmon sampled within the Trinity River.

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INTRODUCTION

Juvenile Klamath River Chinook (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*) experience high incidence and severity of infection with the myxosporean parasites *Ceratomyxa shasta* and *Parvicapsula minibicornis*. Both parasites have a similar distribution and are found throughout the Klamath River system including the lower reaches of the Williamson and Sprague Rivers, Agency Lake, Klamath Lake, Copco Reservoir, and the entire Lower Klamath River from Iron Gate Dam to the estuary (Hendrickson et al. 1989; Stocking et al. 2006; Bartholomew et al. 2007; Stocking and Bartholomew 2007). Both parasites share the vertebrate (salmonid) and invertebrate (*Manayunkia speciosa*) hosts and have overlapping distributions throughout the Pacific Northwest (Ching and Munday 1984; Hoffmaster et al. 1988; Bartholomew et al. 1989; Hendrickson et al. 1989; Bartholomew et al. 1997; Kent et al. 1997; Jones et al. 2004; Bartholomew et al. 2006, Stocking et al. 2006). In previous studies, native Klamath River Salmonids have demonstrated high degrees of *C. shasta* resistance (Foott et al. 1999, Foott et al. 2004; Foott et al. 2007, Stone et al. 2008). Regardless of this resistance, Foott et al. (2004) observed that 100% of Klamath River Chinook salmon became infected and over 80% died within 17d following a 3d exposure in the Klamath River. The observed high incidence of infection in resistant indigenous fish indicates an extremely high parasite challenge (Foott et al. 2004). Dual infections with both parasites are common and have a synergetic effect to increase the lethality of infection (Nichols and True 2007). This study 1) examined the parasite incidence in the juvenile Chinook and coho salmon population throughout the spring out-migration period; 2) compares the pathogen incidence in Iron Gate Hatchery (IGH) and Trinity River Hatchery (TRH) origin Chinook; and 3) compares parasite incidence in 2008 to previous years.

METHODS

Sample Sites

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

Table 1. Sample reach location and cooperating agencies performing collections.

Reach	River Miles	Primary collector(s)
Klamath River mainstem		
IGD to Shasta	Klamath 190-177	USFWS and Karuk Tribe
Shasta to Scott	Klamath 177-143	USFWS and Karuk Tribe
Salmon to Trinity	Klamath 66-44	Karuk Tribe
Trinity to Estuary	Klamath 44-4	Yurok Tribe
Klamath Estuary	Klamath 4-0	Yurok Tribe
Trinity River		
Upper – Lewiston Dam to North Fork	Trinity 111-73	Hoopa Tribe
Lower - North Fork to Klamath	Trinity 73-0	USFWS and Yurok Tribe

QPCR Assay

Fish collected for the quantitative real-time polymerase chain reaction (QPCR) assay were euthanized, placed in a plastic bag labeled with date and reach, and arranged between frozen gel pack sheets in an ice chest. At the end of the day, samples were transferred to a freezer until they could be shipped frozen to the CA-NV Fish Health Center laboratory. In the laboratory, fish were thawed, measured for fork length, and tissue samples were collected. The intestine (both small and large) and kidney tissues from each fish were removed and combined into an individually numbered 2 ml cluster tube. Due to limited tube volume, total sample weight was limited to 1.0g (tissue weight ranged from 0.01g to 1.0g). Tissue samples were then frozen until DNA extraction was performed.

Combined intestine and kidney tissues were digested in 1ml NucPrep Digest Buffer containing 1.25 mg/ml proteinase K (Applied Biosystems, Foster City, CA) at 55ºC for 2 hours with constant shaking. A subsample of digested tissue homogenate was diluted 1:33 in molecular grade water and extracted in a 96 well vacuum filter plate system (Applied Biosystems Model 6100 Nucleic Acid Prep Station). Due to dilution, the weight of tissue entering extraction was limited to 3.0mg given the maximum 1.0g sample weight mentioned above. Extracted DNA was stored at -20ºC until the QPCR assays were performed.

Samples were assayed in a 7300 Sequence Detection System (SDS) (Applied Biosystems), using probes and primers specific to each parasite. The combined tissues were tested for *C. shasta* 18S rDNA using TaqMan Fam-Tamra probe and primers (Hallett and Bartholomew 2006). The combined tissues were also tested for *P. minibicornis* 18S rDNA utilizing TaqMan Minor-Grove-Binding (MGB) probe and primers (True et al. 2009). Reaction volumes of 30µL, containing 5µL DNA template, were used for both assays under the following conditions: 50ºC for 2 min.; 95ºC for 10 min; 40 cycles of 95ºC for 15s and 60ºC for 1 min. Standards, extraction control and no template control wells were included on each assay plate. Cycle threshold (C_T) values were calculated by the SDS software (v 1.3.1, Applied Biosystems). Validation studies examining the dynamic range and endpoint of the assays indicated a C_T of 38 and minimum change in normalized fluorescent signal of at least 10,000 units defines a positive test for the *P. minibicornis* assay (True et al. 2009). Previous assay validation

studies, using DNA plasmid controls and naturally infected fish tissue, determined a similar assay threshold for the *C. shasta* assay. It should be noted that these thresholds are relatively conservative statistically, and therefore slightly underestimate the true infection incidence of both parasites in this aquatic animal population (Appendix 1).

Histology Assay

Fish were fixed in Davidson's fixative in the field and held in fixative for 24-48 hours. Tissues (kidney and intestine) from individual fish were removed, placed into a cassette, and stored in 70% ethanol. Sample were processed for 5μm paraffin sections and stained with hematoxylin and eosin (Humason 1979). All tissues for each fish were placed on one slide and identified by a unique code number. Each slide was examined at 40X to 400X magnification. A composite infection and disease rating was developed based on the degree of tissue inflammation associated with the presence of the parasites. A similar histology rating system has been used in Klamath River monitoring studies since 2004 (Nichols and Foott 2006; Nichols et al. 2007; Nichols and True 2007; Nichols et al. 2008). *Ceratomyxa shasta* infections were rated as clinical (parasite present and inflammatory tissue in >33% of the intestine section), subclinical (parasite present, but inflammatory tissue in <33% of intestine section) or uninfected (no *C. shasta* detected). *Parvicapsula minibicornis* infections were rated as clinical (parasite present and glomerulonephritis in >33% of the kidney section), subclinical (parasite present, and glomerulonephritis in <33% of the kidney section) or uninfected (no *P. minibicornis* detected). Since observations of infections were based on the trophozoite rather than the more diagnostic spore stage of the parasites, diagnosis of *C. shasta* and *P. minibicornis* infections should be considered presumptive and unconfirmed.

Sample Groups

Mixed-origin Chinook – These juvenile Chinook salmon were collected in selected reaches of the Klamath and Trinity Rivers. This sample consisted of 30 Chinook salmon for the QPCR assay and 10 Chinook salmon for the histology assay. In the Shasta to Scott reach and Salmon to Trinity reach of the Klamath River, mixed-origin Chinook were collected every other week. In the upper and lower reaches of the Trinity River, mixed-origin Chinook were collected during the weeks of 11 May, 25 May, 22 June and 6 July. Prior to the release of hatchery fish, these fish were primarily naturally produced with the possibility of a few hatchery origin Chinook salmon used for trap efficiency calibration in the sample. After IGH release, mixed-origin Chinook could have been of either hatchery or natural origin.

Pre-release IGH Chinook salmon - Prior to hatchery release 30 Chinook were sampled from the hatchery population. These Chinook were collected from IGH on 15 May. All 30 pre-release Chinook were assayed by QPCR.

Marked IGH and TRH Chinook salmon - A portion of the Chinook salmon released from Iron Gate Hatchery (IGH) on the Klamath River and Trinity River Hatchery (TRH) on the Trinity River were marked with an adipose fin clip and implanted with a coded-wire-tag (CWT). In the Klamath River, any CWT Chinook encountered by the sample crews were collected for analysis by QPCR. No CWT Chinook were collected in the Trinity

River; however, significant recapture effort occurred in the Klamath River below the Trinity River confluence. Heads from any marked IGH or TRH Chinook salmon recovered were assigned unique identification numbers to track lab assay results to individual fish. Tags were extracted and read by the USFWS Arcata FWO. Chinook salmon were released from IGH on 27 May, 2 June, 9 June, 11 June and 16 June. The CWT codes were different for each release date. A volitional release occurred at TRH, with all CWT codes released at the same time 2 June through 12 June. 7 June was used as the date of release for all marked TRH Chinook salmon. The date each group of CWT Chinook salmon was released from the hatchery and date of recapture was used to calculate weeks at liberty (WAL) for individual fish.

Coho salmon - Juvenile coho salmon encountered in the Klamath River above the Trinity River confluence were collected for the QPCR assay. Coho salmon were collected under endangered species Section 10 permit 1068. No coho salmon were collected in the Trinity River or in the Klamath River below the Trinity River confluence. All young of the year (YOY) coho salmon were of natural origin, and yearling coho salmon may have been natural or hatchery origin.

Sample Periods

In each reach, fish were accumulated over a calendar week until the desired sample size was achieved. Weekly prevalence of infection was calculated for a reach by dividing the number of fish in which a parasite was detected by the total fish assayed for a calendar week. Fish collection started the week of 20 April in the Shasta to Scott reach and 4 May in the Salmon to Trinity reach. Collection continued until the minimum Chinook salmon sample numbers per week (10 fish) could no longer be captured. Collection of CWT Chinook salmon began after hatchery release and collection crews would accumulate as many CWT Chinook salmon as time allowed each week. Collection of CWT Chinook salmon in a given reach continued until fewer than 10 fish could be recovered in a week's effort.

2004-2008 Comparisons

Histology data from this and previous juvenile Klamath River salmonid health monitoring studies (Nichols and Foott 2006; Nichols et al. 2007; Nichols and True 2007; Nichols et al. 2008) was used to compare total incidence and severity of infection of fish in 2008 to previous years (2004-2007). While QPCR data was available for 2005-2008 extraction methods have been optimized during that period making that data unsuitable for comparison between years. The histology data included in the analysis was limited to the months of May, June and July of each year and to mixed-origin Chinook sampled in the Klamath River above the Trinity River confluence. Limiting the data offered several advantages:

- Sampling start and end dates varied each year but included these months
- This date range brackets the typical peak of juvenile Klamath River Fall Chinook Salmon outmigration (Leidy and Leidy 1984; Wallace and Collins 1997)
- Infection incidence during the "tails" of the migration (typically lower infection rates in early spring) were not given the same weight as the peak of migration
- The Trinity River population was excluded as it is largely *C. shasta* uninfected

- Our target sample size was typically met during this period reducing sample variation due to small sample size

RESULTS

Mixed-origin Chinook

In the Shasta to Scott reach, *C. shasta* was detected by QPCR in 60% (114/191, 95% confidence interval [ci]=52-67%) and by histology in 43% (23/54, ci=29-57%) of mixed-origin Chinook. Infection prevalence peaked at over 80% in mid May and was possibly peaking again when sampling in this reach ended in late June (Figures 1 and 2). Infection severity peaked in mid May and was increasing again at the last sample in late June (Figure 2).

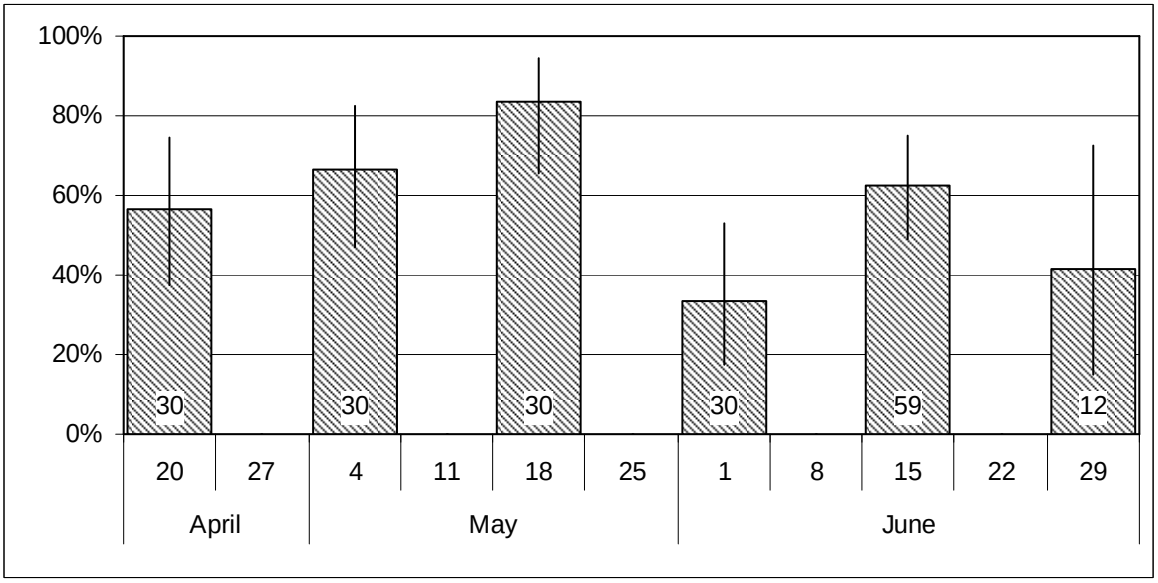

Figure 1. Biweekly incidence of *Ceratomyxa shasta* infection assayed by QPCR in Klamath River Chinook salmon captured in the Shasta to Scott reach. Whiskers indicate 95% confidence interval and sample number is at base of each bar.

In the Shasta to Scott reach, *P. minibicornis* was detected by QPCR in 80% (153/191, ci=74-86%) and by histology in 86% (44/51, ci=74-84%) of mixed-origin Chinook. Infection incidence reached 100% in mid May and remained high through the end of sampling in this reach in late June (Figures 3 and 4). Infection severity peaked in early June with 90% of fish with clinical infections (Figure 4).

In the Salmon to Trinity reach, *C. shasta* was detected by QPCR in 37% (100/270, ci=31-43%) and by histology in 26% (21/80, ci=17-37%) of mixed-origin Chinook. Weekly infection prevalence peaked during June then dropped to near zero by the last sample from this reach in early August (Figures 5 and 6). Infection severity also peaked during June and tended to drop in July and August (Figure 6).

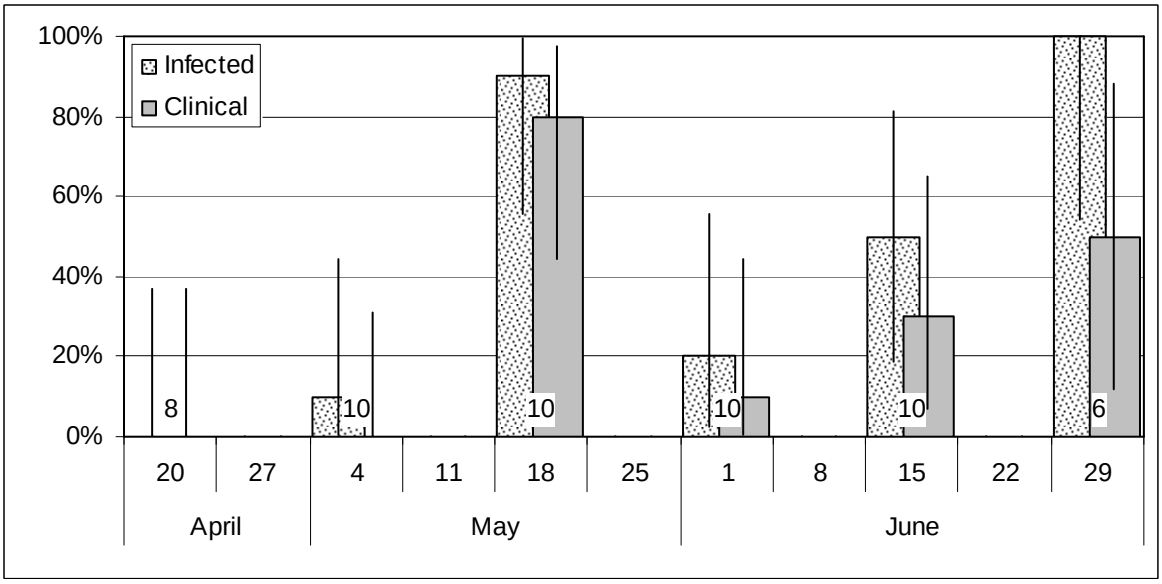

Figure 2. Biweekly incidence of *Ceratomyxa shasta* infection and significant intestinal lesion (Clinical) assayed by histology in Klamath River Chinook salmon captured in the Shasta to Scott reach. Whiskers indicate 95% confidence interval and sample number is at base of each bar.

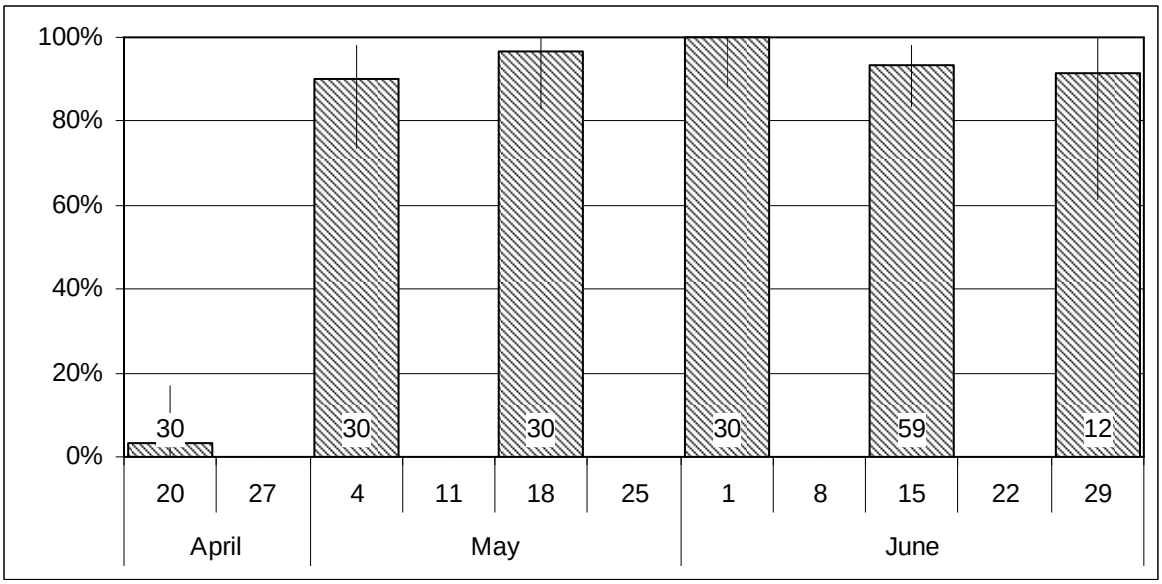

Figure 3. Biweekly incidence of *Parvicapsula minibicornis* infection assayed by QPCR in Klamath River Chinook salmon captured in the Shasta to Scott reach. Whiskers indicate 95% confidence interval and sample number is at base of each bar.

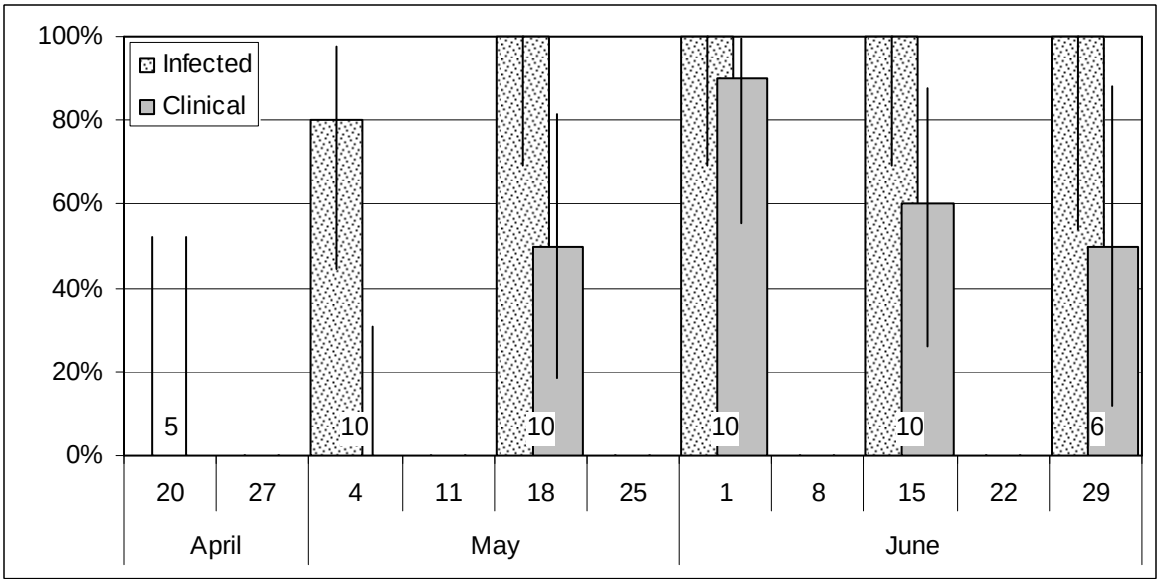

Figure 4. Biweekly incidence of *Parvicapsula minibicornis* infection and significant intestinal lesion (Clinical) assayed by histology in Klamath River Chinook salmon captured in the Shasta to Scott reach. Whiskers indicate 95% confidence interval and sample number is at base of each bar.

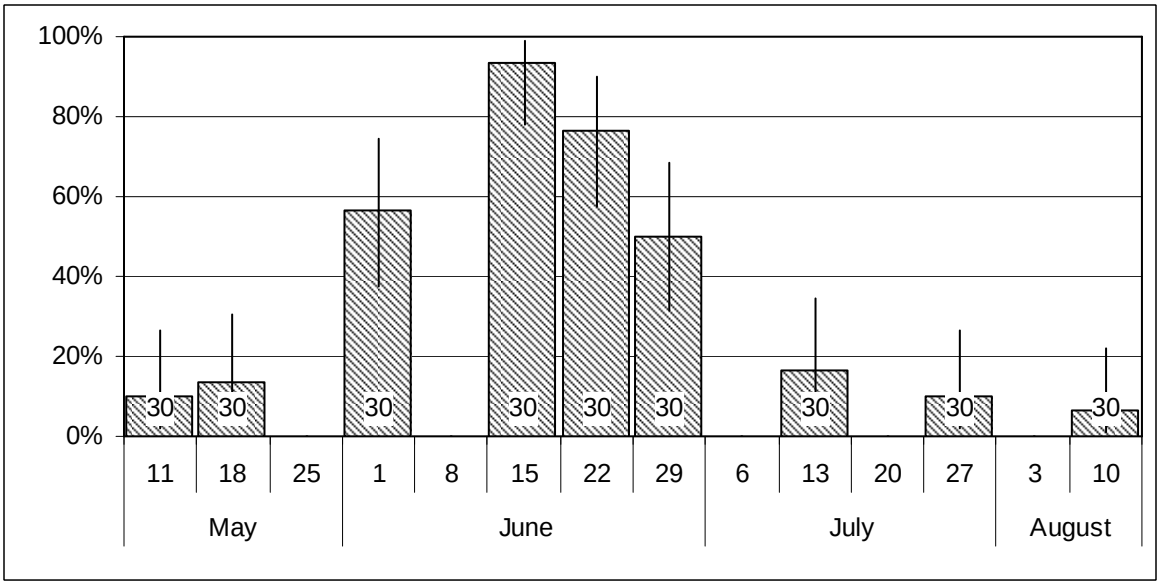

Figure 5. Biweekly incidence of *Ceratomyxa shasta* infection assayed by QPCR in Klamath River Chinook salmon captured in the Salmon to Trinity reach. Whiskers indicate 95% confidence interval and sample number is at base of each bar.

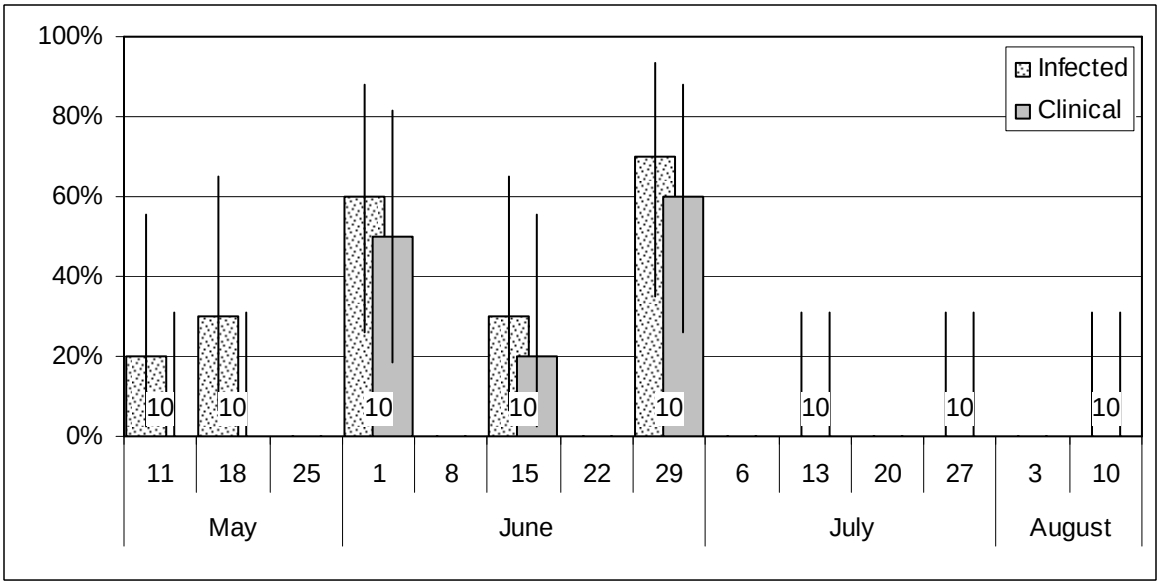

Figure 6. Biweekly incidence of *Ceratomyxa shasta* infection and significant intestinal lesion (Clinical) assayed by histology in Klamath River Chinook salmon captured in the Salmon to Trinity reach. Whiskers indicate 95% confidence interval and sample number is at base of each bar.

In the Salmon to Trinity reach, *P. minibicornis* was detected by QPCR in 85% (229/270, ci=80-89%) and by histology in 85% (68/80, ci=75-92%) of mixed-origin Chinook. Weekly infection prevalence peaked during June then remained high through early August in these fish (Figures 7 and 8). Infection severity also peaked at 90% during June and dropped in July and August (Figure 8).

In the Trinity River, *C. shasta* was detected by QPCR in 1% (2/158, ci=0-5%) and by histology in none (0/30, ci=0-12%) of the mixed-origin Chinook. One infected Chinook salmon was captured in the upper Trinity River reach and the other in the lower Trinity River reach. Both infected fish had light infections near the detection limit of the QPCR assay (data not presented).

In the Trinity River, *P. minibicornis* was detected by QPCR in 6% (9/158, ci=3-11%) and by histology in none (0/30, ci=0-12%) of the mixed-origin Chinook. Infected Chinook salmon were found in both the upper and lower Trinity River reaches. Infected fish had light to moderate infections with the majority of infections near the QPCR assay detection cutoff (data not presented).

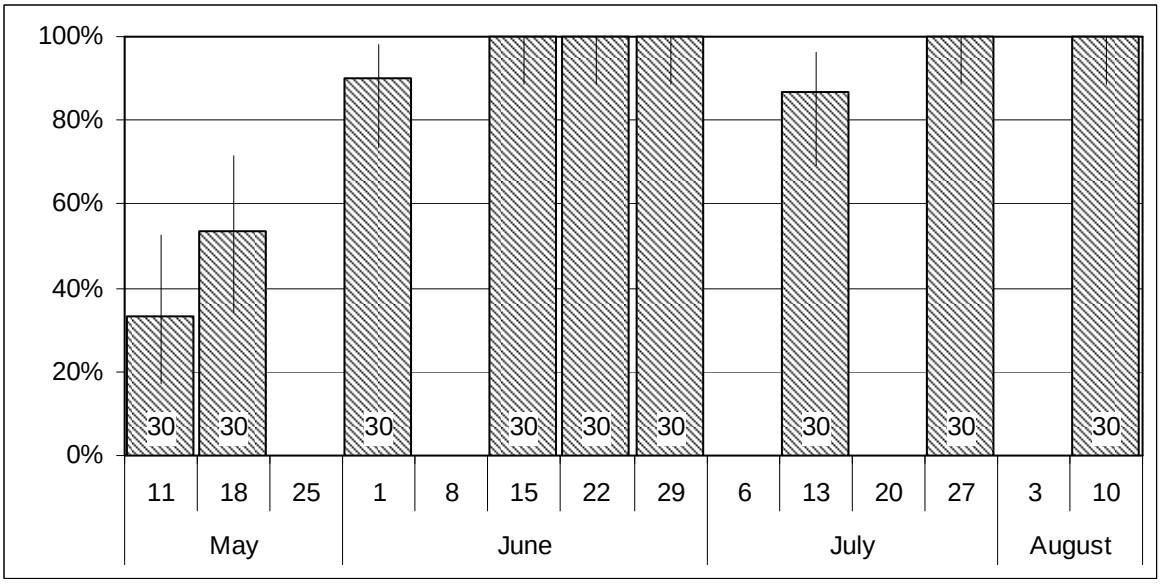

Figure 7. Biweekly incidence of *Parvicapsula minibicornis* infection assayed by QPCR in Klamath River Chinook salmon captured in the Salmon to Trinity reach. Whiskers indicate 95% confidence interval and sample number is at base of each bar.

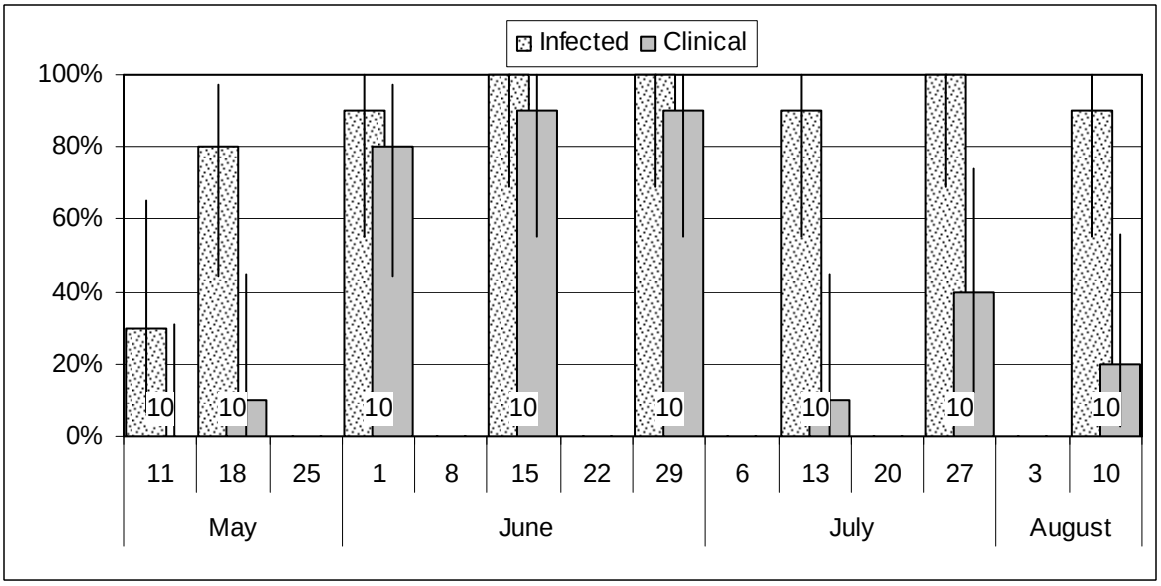

Figure 8. Biweekly incidence of *Parvicapsula minibicornis* infection and significant intestinal lesion (Clinical) assayed by histology in Klamath River Chinook salmon captured in the Salmon to Trinity reach. Whiskers indicate 95% confidence interval and sample number is at base of each bar.

Pre-release IGH Chinook Salmon

Light infections of *C. shasta* were detected by QPCR in 3% (1/30, ci=0-17%) of Chinook salmon sampled at IGH prior to the release. The single fish was lightly infected near the infection threshold of the QPCR assay (data not presented). Infections of *P. minibicornis* were detected by QPCR in 3% (1/30, ci=0-17%) of pre-release Chinook salmon sampled

at IGH. The single infection detected indicated a moderate level of parasite DNA (data not presented).

Marked IGH Chinook Salmon

Ceratomyxa shasta was detected in 27% (32/110, ci=21-39%) of the marked IGH Chinook salmon screened by QPCR. No infections were detected in the week after hatchery release, but *C. shasta* weekly infection prevalence peaked at 69% (22/32, ci=50-84%) by the third week follow release from IGH (Figure 9). Infections were detected in marked IGH Chinook salmon recovered out to 6 WAL, and no infections were detected in the fish recovered 7 to 10 WAL. When examined by reach in which fish were recovered, infection incidence in marked IGH Chinook salmon peaked at 69% in the Shasta to Scott reach and declined downstream with no infections detected in the 9 fish recovered in the estuary (Figure 10).

Parvicapsula minibicornis infections were detected in 72% (86/119, ci=63-80%) of the marked IGH Chinook salmon screened for by QPCR. No infections were detected in the week after hatchery release, but weekly infection prevalence reached 100% (32/32, ci=89-100%) by the third week follow release from the hatchery (Figure 11). Infections were detected in smolts recovered through 10 WAL. When examined by reach in which fish were recovered infection incidence was high in both the Shasta to Scott reach (92%) and Trinity to Estuary reach (95%) and declined in Marked IGH Chinook salmon recovered in the estuary (Figure 12).

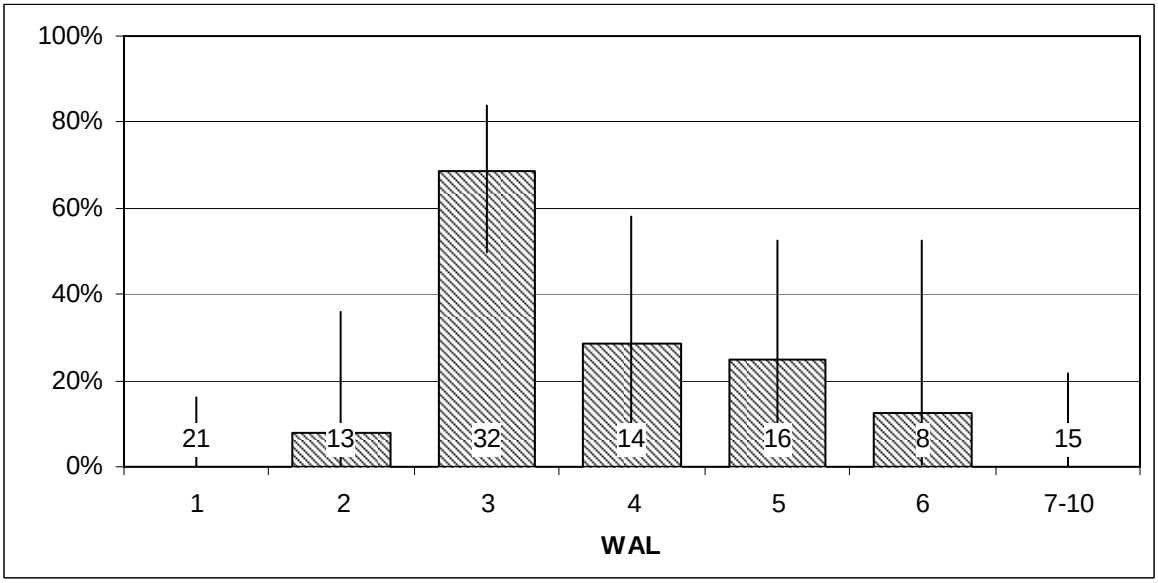

Figure 9. *Ceratomyxa shasta* incidence of infection assayed by QPCR in CWT Iron Gate Hatchery Chinook recovered in the Klamath River by weeks since hatchery release (WAL). Whiskers indicate 95% confidence interval and sample number is at the base of each bar.

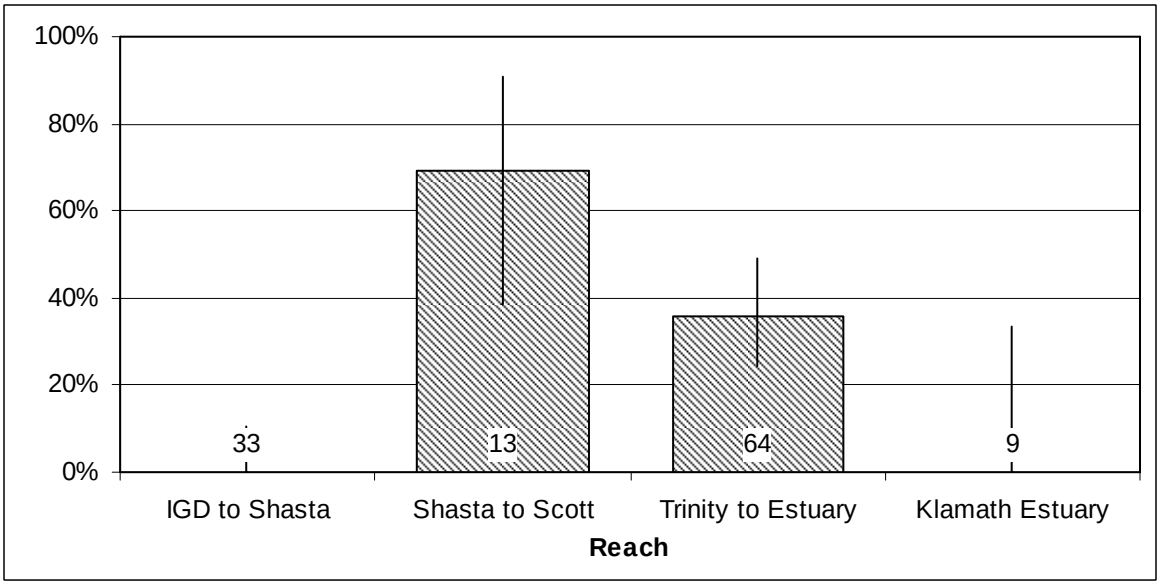

Figure 10. *Ceratomyxa shasta* incidence of infection assayed by QPCR in CWT Iron Gate Hatchery Chinook recovered in the Klamath River by reach where fish were recovered. Whiskers indicate 95% confidence interval and sample number is at the base of each bar.

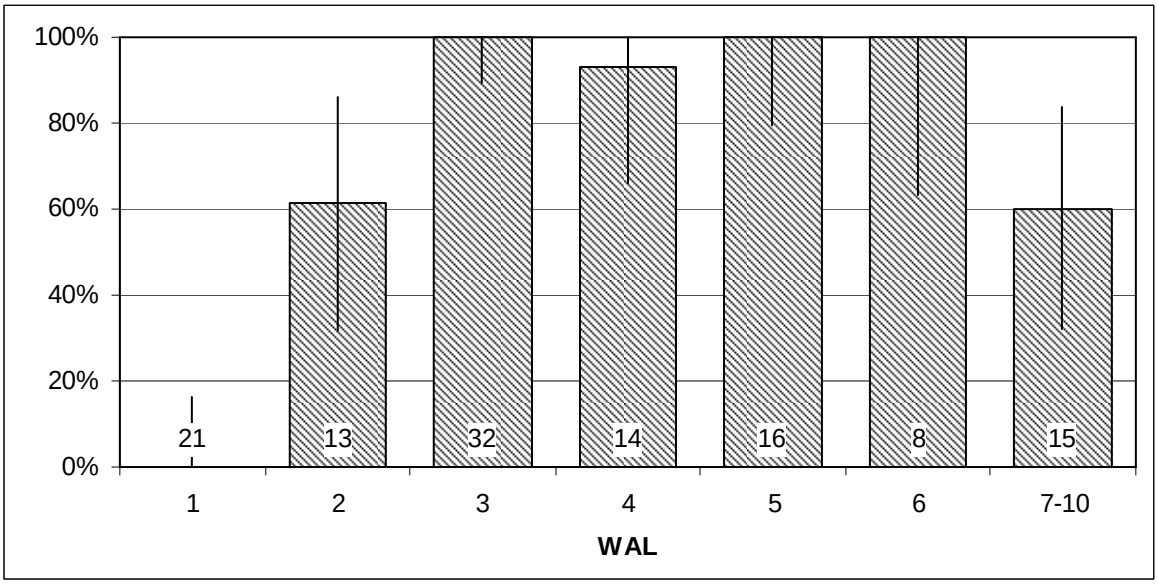

Figure 11. *Parvicapsula minibicornis* incidence of infection assayed by QPCR in CWT Iron Gate Hatchery Chinook recovered in the Klamath River by weeks since hatchery release (WAL). Whiskers indicate 95% confidence interval and sample number is at the base of each bar.

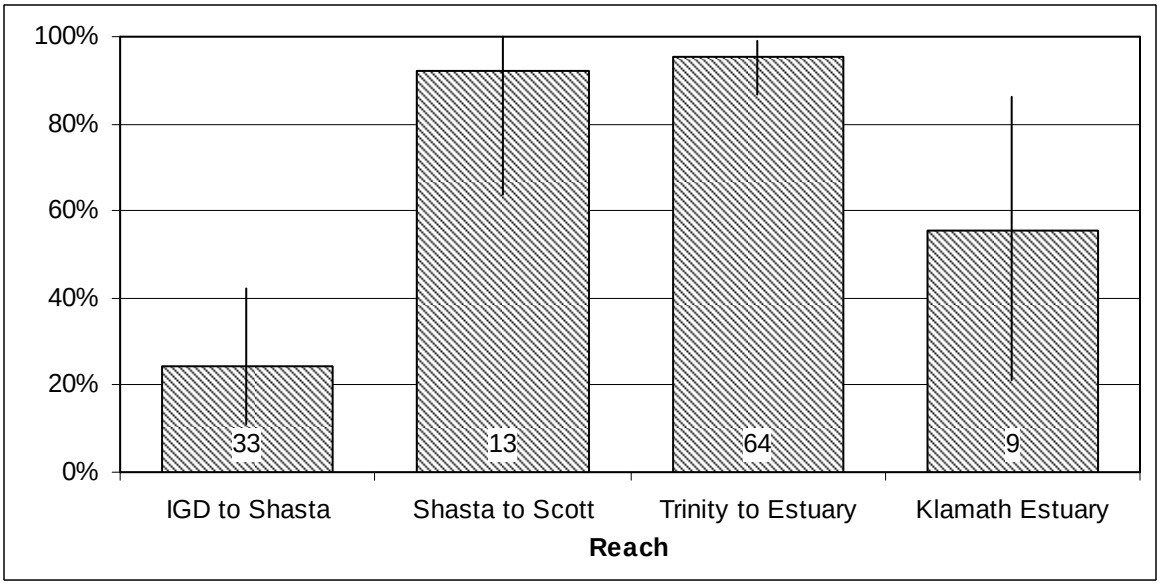

Figure 12. *Parvicapsula minibicornis* incidence of infection assayed by QPCR in CWT Iron Gate Hatchery Chinook recovered in the Klamath River by reach where fish was recovered. Whiskers indicate 95% confidence interval and sample number is at the base of each bar.

Marked TRH Chinook salmon

Ceratomyxa shasta was detected in 3% (8/257, ci=1-6%) of the marked TRH Chinook salmon screened by QPCR. No infections were detected in the marked TRH Chinook salmon recovered in the Klamath River until the fifth WAL, and infections were detected through 12 WAL (Figure 13).

Parvicapsula minibicornis was detected in 35% (97/281, ci=29-40%) of the marked TRH Chinook salmon screened by QPCR. Infections were not detected until 5 WAL, peaked first at 43% in the sixth WAL, and again at 84% 12 WAL (Figure 14).

Coho salmon

Ceratomyxa shasta was detected by QPCR in 6% (2/33, ci=1-20%) of the YOY coho salmon and 29% (2/7, ci=4-71%) of the yearling coho salmon. *Parvicapsula minibicornis* was detected by QPCR in 11% (4/37, ci=3-25%) of YOY coho salmon and 63% (5/8, ci=24-91%) of the yearling coho salmon.

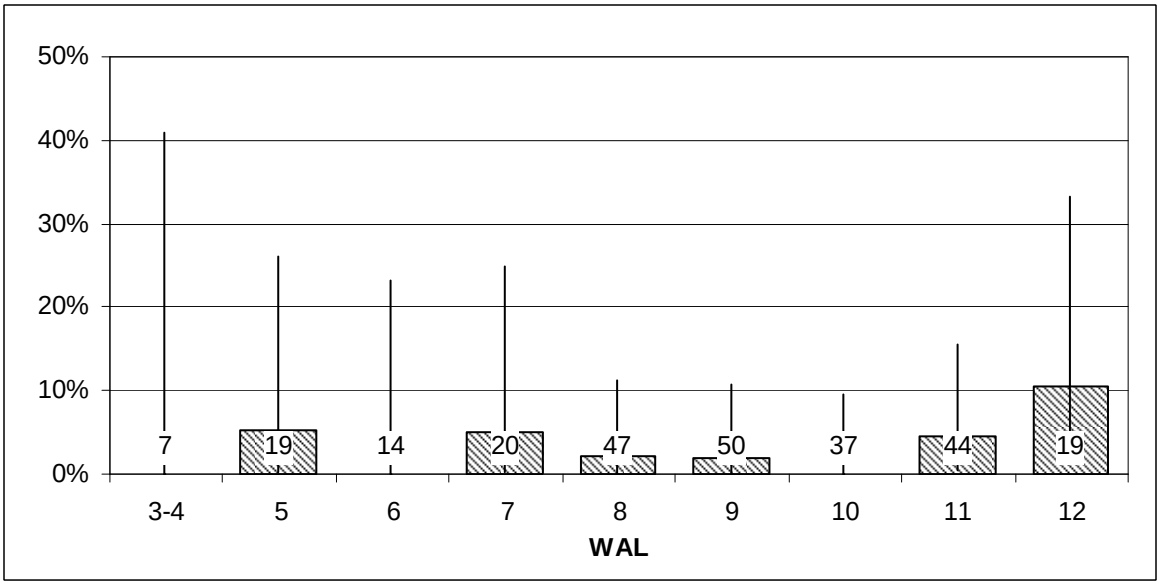

Figure 13. *Ceratomyxa shasta* incidence of infection assayed by QPCR in CWT Trinity River Hatchery Chinook salmon recovered in the Klamath River by weeks since hatchery release (WAL). Whiskers indicate 95% confidence interval and sample number is at the base of each bar.

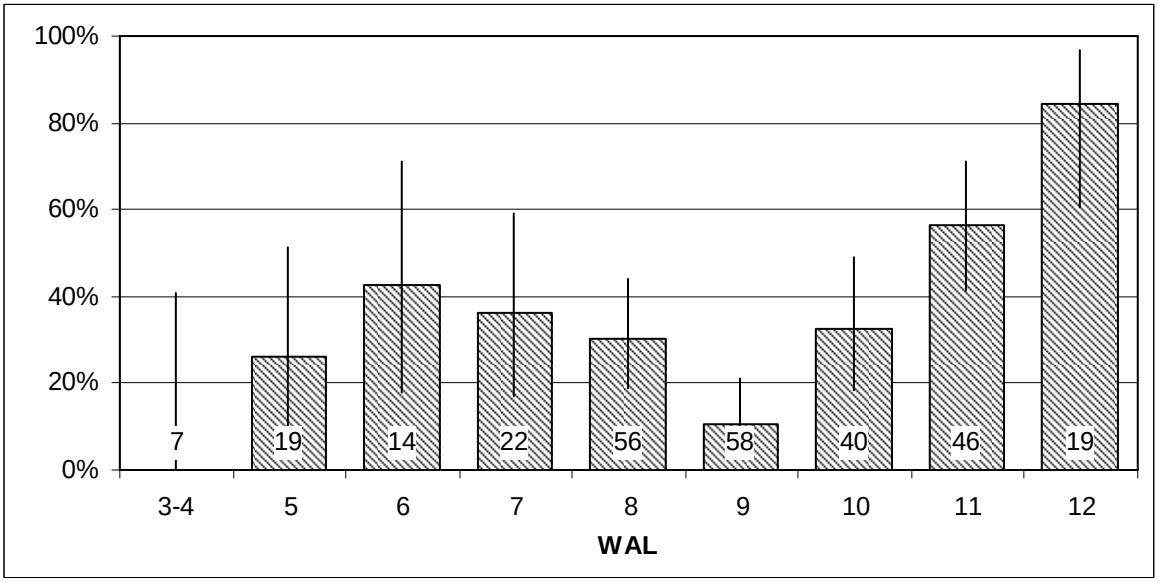

Figure 14. *Parvicapsula minibicornis* incidence of infection assayed by QPCR in CWT Trinity River Hatchery Chinook salmon recovered in the Klamath River by weeks since hatchery release (WAL). Whiskers indicate 95% confidence interval and sample number is at the base of each bar.

2004-2008 Comparisons

The incidence of *C. shasta* by histology in mixed-origin Chinook captured during May, June and July in the Klamath River above the confluence of the Trinity River was 38% (44/116, ci=29-47%). Compared to studies in previous years using similar methods,

2008 *C. shasta* incidence of infection and rate of severe (clinical) infections in mixed-origin Chinook was high, with levels similar to those seen in 2004 and 2005 (Table 2).

The incidence of *P. minibicornis* in mixed-origin Chinook during May, June and July captured in the Klamath River above the confluence of the Trinity River was 89% (103/116, ci=82-94%). Compared to previous studies using similar methods the total incidence of *P. minibicornis* infection in mixed-origin Chinook was higher than average, with levels similar to those observed in Chinook salmon sampled in 2005 (Table 3). The incidence of severe (clinical) *P. minibicornis* infections were near the 2004-2008 average, and lower than observed in 2005.

Table 2. Comparison of *Ceratomyxa shasta* incidence in juvenile Klamath River Chinook salmon from 2004-2008 assayed by histology. Percentages indicate proportion of the total samples (N) in which the parasite was detected (Incidence) or had an intestinal lesion associated with an infection (Clinical). The 95% confidence interval is presented in brackets. Only fish sampled during May-July and captured in the Klamath River above the Trinity River confluence were included to aid comparisons between years.

	2004	2005	2006	2007	2008	Average 2004-2008
Incidence	34% [31-38]	35% [27-44]	21% [14-30]	21% [13-31]	38% [29-47]	30%
Clinical	23% [20-26]	21% [14-29]	18% [11-26]	15% [8-25]	24% [17-33]	20%
N	735	134	112	81	116	n/a

Table 3. Comparison of *Parvicapsula minibicornis* incidence in juvenile Klamath River Chinook salmon from 2004-2008 assayed by histology. Percentages indicate proportion of the total samples (N) in which the parasite was detected (Incidence) or had an intestinal lesion associated with an infection (Clinical). Only fish sampled during May-July and captured in the Klamath River above the Trinity River confluence were included to aid comparisons between years.

	2004	2005	2006	2007	2008	Average 2004-2008
Incidence	77% [74-80]	92% [86-96]	58% [48-67]	81% [71-89]	89% [82-94]	79%
Clinical	37% [33-41]	65% [56-73]	29% [21-38]	53% [42-64]	47% [38-57]	46%
N	731	134	112	81	116	n/a

DISCUSION

The incidence of both *C. shasta* and *P. minibicornis* infections in juvenile Chinook salmon has been monitored in fish health studies in the Klamath River since 2004 (Nichols and Foott 2006; Nichols et al. 2007; Nichols and True 2007; Nichols et al. 2008). The May-July 2008 incidence of *C. shasta* by histology was greater than levels observed during the previous two years, and the incidence of *P. minibicornis* by histology in Chinook salmon was similar to the record levels seen in 2005. The peak weekly prevalence of *C. shasta* infections in mixed-origin Chinook sampled in the Shasta to Scott reach was observed prior to the release of IGH Chinook salmon. As hatchery

releases did not begin until 27 May, Chinook captured prior to hatchery release were all considered to be naturally produced. The weekly prevalence of infection in naturally produced Chinook prior to hatchery release peaked at over 80%. It was hypothesized that this high infection rate was a result of these Chinook salmon residing in highly infectious waters, and that naturally produced Chinook and coho salmon that rear in the mainstem Klamath River have a greater probability to incur disease mortality than tributary fish which spend less time the mainstem Klamath River.

Infection incidence declined in Chinook salmon captured late in the study during the months of July and August. Factors which could contribute to these lower levels of infection include: mortality of infected fish, survival of the most resistant fish, and the influx of uninfected hatchery and tributary fish. Evidence that some fish were able to limit their exposure to infection and disease was observed in the mixed-origin Chinook captured during July and August in the Salmon to Trinity reach. These fish had lower levels of *C. shasta* infection and fewer clincial infections of *P. minibicornis* compared to fish captured a few weeks earlier. Unfortunately, these fish were not marked, so their origin was unknown.

Incidence of *P. minibicornis* in mixed-origin Chinook remained high following hatchery release which was the same trend observed in previous years (Nichols and Foott 2006; Nichols et al. 2007; Nichols and True 2007; Nichols et al. 2008). In these previous studies, *P. minibicornis* has consistently proven to maintain high levels of infection even when rates of *C. shasta* infection drop. Unfortunately, the prognosis of these *P. minibicornis* infections in juvenile Chinook is not well studied. The synergistic effect of dual *C. shasta* and *P. minibicornis* infections likely increased the risk of lethal disease (Nichols and True 2007).

The marked IGH and TRH Chinook salmon sampled demonstrated that the Klamath River reaches above the Trinity River confluence were highly infectious, while below the Trinity River confluence infections were less common, particularly for *C. shasta*. While low incidences of infection (3%) for both parasites were detected in pre-release IGH sampling, no parasites were detected in the earliest marked IGH Chinook salmon recovered after release from the hatchery. The incidence of infection for both parasites increased rapidly in marked IGH Chinook salmon when they were recaptured in the highly infectious Shasta to Scott reach (Stocking et al. 2006). The decreased incidence of infection in marked IGH Chinook salmon below the Trinity River confluence suggests infected fish had succumbed to disease above that point. Those marked IGH Chinook salmon which survived to the estuary were perhaps more resistant to infection or limited their exposure to infection by migrating rapidly through the highly infectious zone. Marked IGH Chinook salmon can be viewed as surrogates for demonstrating the disease impacts on naturally produced Chinook salmon emigrating from Shasta and Scott Rivers.

The incidence of *C. shasta* infection remained low in marked TRH Chinook salmon sampled in the Klamath River and was similar to the 1% incidence observed in mixed-origin Chinook sampled in the Trinity River. The incidence of *P. minibicornis* infection in marked TRH Chinook salmon increased with time following hatchery release, but was

lower than infection incidence observed in marked IGH Chinook salmon that had passed though highly infectious reaches of the Klamath River.

Only a few juvenile coho were captured for this study, and it was difficult to make conclusions based on this small sample size. Incidence for both parasites appeared lower in coho salmon than Chinook salmon, and yearling coho salmon likely had a greater incidence of infection than YOY coho salmon in 2008. Parasite incidence in coho salmon has varied in past Klamath River fish health monitoring studies; with coho salmon having a higher incidence of infection than Chinook salmon in some years (Nichols and True 2007; Nichols et al. 2008). While coho salmon appeared to have faired better than Chinook salmon in 2008, there may not be a large difference between the two species in parasite susceptibility (Stone et al. 2008).

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AUTHOR ROLES

The contributions of each author have been summarized below.

- Ken Nichols – project coordination, study design, data management, histology analysis, assembly and editing of final report
- Kimberly True – QPCR methods and assay analysis, DNA extraction QA
- Ryan Fogerty – Necropsy, field collection coordination and histology processing
- Lisa Ratcliff – Necropsy, DNA extraction and QPCR assay
- Anne Bolick - DNA extraction and QPCR assay

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APPENDIX I
Reviewers' comments

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

Title

Comment 1. Title of the report suggests a larger focus than just the examination of the parasite incidence in the out-migrant Chinook and coho salmon population throughout the spring out-migration period. Based on the stated objectives, I suggest a narrower title than - Klamath River Juvenile Salmonid Health Monitoring. Health would include other aspects of the fish besides parasite incidence.

Reply: Title was changed to reflect study's emphasis.

Comment 2. From what follows, I understand Chinook and coho to be the only species referred to here. Other "Klamath River salmonids" would include steelhead, cutthroat (in Lower Klamath) and the non-native brown trout (abundant near Lewiston on the Trinity).

Reply: Text was changed to better reflect the species.

Summary

Comment 3. With the small coho salmon sample size and limited distribution of the samples, making conclusions or comparisons in regards to coho salmon infection is questionable.

Reply: Due to the uncertainty associated with the small sample size this statement was removed from the summary. Results and discussion sections already reflect the small sample size and were unchanged.

Introduction

Comment 4. Citations needed: "It is assumed that these infected fish incur elevated mortality associated with disease during the spring out-migration."

Reply: Subject was address with a citation later in the Introduction. This statement was removed.

Methods

Comment 5. Here, or where appropriate, please discuss the implications of the earlier statement "Since observations of infections were based on the trophozoite rather than the more diagnostic spore stage of the parasites, diagnosis of *C. shasta* and *P. minibicornis* infections should be considered presumptive and unconfirmed."

Reply: This is a limitation of the chosen assay. The implications were beyond the scope of this report; however the use of the QPCR assays in conjunction with histology offers some general confirmation of the histology findings. These assays were not performed on the same fish so no direct confirmation was performed. The text was not changed.

Results

Comment 6. A summary table with the point estimate and the 95 confidence level corresponding to the figures would have assisted the reader.

Reply: Confidence intervals were added to Tables 2 and 3.

Comment 7. Most results are Chinook salmon specific. The text within the document needs to better identify which species is being discussed. For example a smolt may be either Chinook or coho salmon.

Reply: The text was changed to better identify the sample group in question.

Comment 8. The 95 percent confidence range should be reported when giving results. The use of the 95 percent confidence level should also be used in making conclusions or in comparisons. I would conjecture that when the confidence level is taken into consideration, that the *C. Shasta* incidence level between 2004 through 2008 are very similar.

Reply: CI added and conclusions reflect significant difference found when appropriate.

Comment 9. Clarify QPCR is not appropriate for between years

Reply: While QPCR data was available for 2005-2008 extraction methods have been optimized during that period making that data unsuitable for comparison between years. This was clarified in the text.

Discussion

Comment 10. The use of "near the highest level" and "suffer severe infection" should be quantified. Do the three month estimates for each year have an associated confidence level? If so, those levels should also be provided. I would conjecture that when the confidence level is taken into consideration, that the *C. Shasta* incidence level between 2004 through 2008 are very similar.

Reply: High is a relative term and compares 2008 to previous years. The text was changed to identify previous years which were similar. The use of "severe" was changed to "clinical" which was defined in the Methods.

Comment 11. The document contains numerous speculations that are not supported by the information presented. Either present the information necessary to come to the given assumption or remove.

Reply: These statements were offered as a possible explanation of the data. Where needed the statements were clearly identified as hypothesis or eliminated.

Comment 12. Please provide the reader what is the "typical outmigration period."

Reply: This phrase was used excessively and text was changed to better define the time period.

Comment 13. Hatchery smolts were marked by an adipose fin clip and implanted code-wire-tag. With the increased stress associated with marking, including open wounds, when compared to the natural population, it is possible that marked hatchery fish may be more susceptible to infection.

Reply: While it has been demonstrated that marking fish can spread pathogens. The fish had healed by time of release. Also, in this study CWT marked Chinook may have had a lower overall incidence of infection compared to mixed-origin Chinook.

Comment 14. Not clear what is meant by this term, "random Chinook". I am inclined to understand this as observations of Chinook sampled casually, outside the study design, and before the onset of the defined "outmigration period".

Reply: The "random Chinook" sample group has been changed to "mixed-origin Chinook". The sample group definition in the Methods section was also changed to better define this group of fish.

Comment 15. Needs citation: The synergistic affect of dual *C. shasta* and *P. minibicornis* infections likely increased the risk of lethal disease.

Reply: This is an hypothesis presented in much of the fish health work done on the Klamath River. Detail was presented in Nichols and True (2007) so that report was referenced.

Comment 16. How did the study determine how long the [TRH] smolt spent in the Klamath River?

Reply: The text was changed to specify release from TRH as the starting time point.

Comment 17. Could not the smolt that "rapidly migrated" through the upper reaches had a reduced chance of capture, thus being sampled. This could also explain the decreased incidence of infection in IGH smolts below the Trinity confluence.

Reply: A rotary screw trap was the primary method of capture in the Shasta to Scott, and we believe that all fish had an equal chance of capture as they migrated past the trap.

Comment 18. The small coho salmon sample size and limited distribution of the coho salmon samples, making conclusions or comparisons for coho salmon is questionable. The Summary at top of report should reflect this comparative uncertainty of coho findings

Reply: This limitation was presented at the beginning of discussion section on coho salmon. Since work was done on coho for this study some discussion of the results was needed even with the limitations of the sample size. Mention of coho salmon results has been removed from the Summary due to the limitation of the data.

Comment 19. Do you mean to compare non-Klamath fish with Klamath fish when discussion "fish which do not rear in the Klamath", or are you contrasting naturally produced Klamath fish with hatchery produced Klamath fish?

Reply: The phrase was attempting to describe the length of time spend in the Klamath and exposed to the infectious stage of the parasites. The text has been changed to specify time.

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

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

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

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

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

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

It should be noted that when the assay threshold conforms to the statistically valid standard deviation of 0.30, a small proportion of test samples that contain very low copy numbers of parasite DNA may be excluded from the positive test group and prevalence data set (false negative or Type II error). Conversely, if larger standard deviation values are chosen to establish the assay positive threshold, a small proportion of false-positive samples would be included in the prevalence data set (Type I error). For the Klamath monitoring program, we have followed the instrument manufacturer's recommendation regarding assay threshold to preclude the inclusion of false-positive test results. We believe the small proportion of fish, with extremely low parasite DNA levels, are not biologically significant in terms of disease risk, nor in reporting the overall prevalence of infection for this parasite. The *Pm* QPCR assay positive threshold precludes false-

positive test results from the apparent prevalence data and therefore is conservative in estimating the true prevalence of disease in this aquatic animal population.