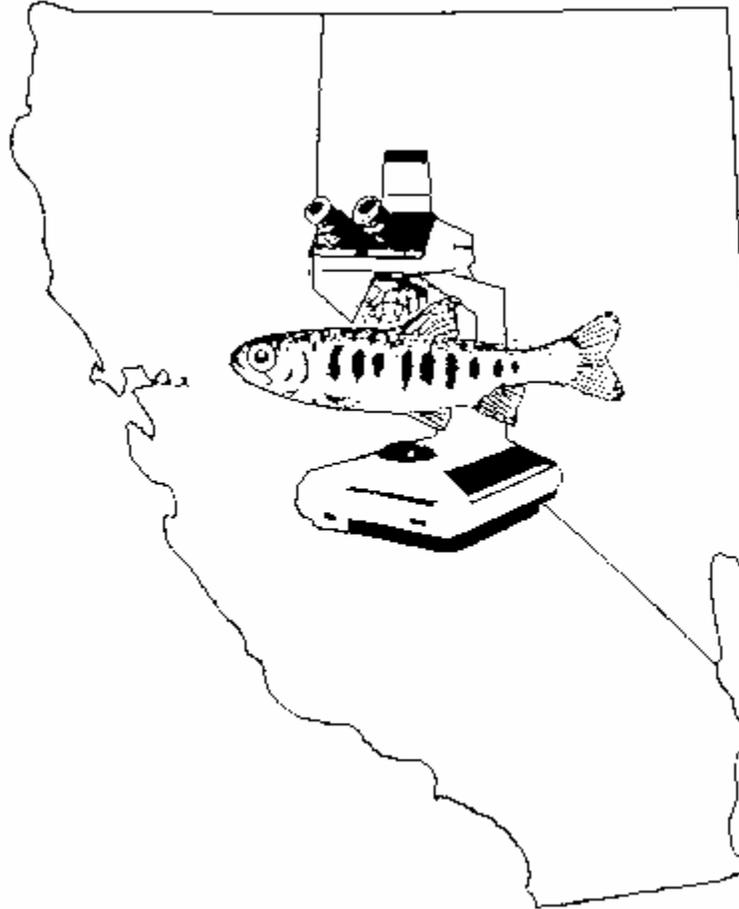


FY 2005 Investigational Report:  
**INCIDENCE OF *CERATOMYXA SHASTA* AND *PARVICAPSULA MINIBICORNIS* INFECTIONS BY QPCR AND HISTOLOGY IN JUVENILE KLAMATH RIVER CHINOOK SALMON**



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## Summary

In this study we monitored the weekly incidence of infection for two myxosporean parasites (*Ceratomyxa shasta* and *Parvicapsula minibicornis*) in juvenile Klamath River Chinook Salmon (*Onchorynchus tshawycha*) over 19 weeks (March – July) during the spring 2005 out-migration. Sites were sampled on the Klamath River from just below Iron Gate Dam, the upper limit of anadromous migration, to the Klamath Estuary and at one site in the lower Trinity River. We utilized Quantitative real-time Polymerase Chain Reaction (QPCR) to increase our sensitivity and efficiency for detection of *C. shasta* and *P. minibicornis*. The QPCR assay amplifies specific sequences of DNA to identify the presence of the target organism (or parasite). A subset of fish was assayed by both histology and QPCR to compare the methods, and provide continuity with previous studies. The *C. shasta* incidence of infection observed during May-July 2005 (62% by QPCR and 35% by histology) was similar to that observed in previous studies. The *P. minibicornis* infection during May-July 2005 (92% by histology and 96% by QPCR) was at the upper range seen in past years. The high prevalence of *P. minibicornis* infections results in nearly all (98%) of the *C. shasta* infected fish having dual infections.

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## Notice

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## INTRODUCTION

The California-Nevada Fish Health Center, as a partner in the efforts to restore salmonid populations in the Klamath River basin, has conducted pathogen monitoring of juvenile Klamath River Chinook Salmon (*Oncorhynchus tshawytscha*) since 1991. Pathogens associated with diseased fish in the Klamath River include bacteria (*Flavobacterium columnare* and motile aeromonid bacteria), digenetic trematode (presumptive *Nanophyetus salmincola*) and myxozoan parasites (*Parvicapsula minibicornis* and *Ceratomyxa shasta*). Ceratomyxosis (due to *C. shasta*) has been identified as the most significant disease for juvenile salmon in the Klamath Basin (Foott et al. 1999; Foott et al. 2004). Significant kidney damage (glomerulonephritis) has been associated with *P. minibicornis* infection; however, the prognosis of such infections has not been studied.

*Ceratomyxa shasta* and *Parvicapsula minibicornis* are myxosporean parasites found in a number of Pacific Northwest watersheds (Bartholomew et al. 1997; St. Hilare et al. 2002; Jones et al. 2004; Bartholomew et al. 2006). The lifecycles of both parasites include the polychaete host, *Manayunkia speciosa*, and salmonids (Hoffmaster et al. 1988; Bartholomew et al. 1997; Bartholomew et al. 2006). *Ceratomyxa shasta* infection can occur from spring through fall at water temperatures > 4°C (Ching and Munday 1984; Hendrickson et al. 1989; Bartholomew et al. 1989). Studies conducted in 2004 and 2005 suggest that *P. minibicornis* has seasonality similar to that of *C. shasta*, while its actinospore concentration and infectivity appears greater than *C. shasta* (Foott et al. 2006; Nichols and Foott 2006).

In this study we monitored the weekly incidence of *C. shasta* and *P. minibicornis* infection in juvenile Chinook salmon over 19 weeks of the spring out-migration period. Previous studies have relied on the observation of the parasites by histology or wet mount preparations. This study utilized Quantitative real-time Polymerase Chain Reaction (QPCR) to increase our sensitivity and efficiency for detection of *C. shasta* and *P. minibicornis*. The QPCR assay amplified specific sequences of DNA to identify the presence of the target. A subset of fish was assayed by both histology and QPCR to compare the methods, and provide continuity with previous studies.

## METHODS

### Collection and Necropsy

Sample Collection – Sites and methods are summarized in Table 1 and Figure 1. Sites were combined into sample reaches separated by major tributaries. Using reaches allowed our data to fit with existing water quality sampling strategies and permitted us to move and combine nearby sites as river conditions change.

The US Fish and Wildlife Service, Yurok Tribal Fisheries and Karuk Tribal Fisheries field crews collected fish and randomly selected 30 live fall-run Chinook salmon from each sample site. Fish were euthanized in MS222, measured for fork length and examined for abnormalities. Tissue samples were collected for QPCR and histology assays.

Tissue collection for QPCR – Approximately 0.05g of kidney and 0.05g of intestine were removed from each fish. The entire kidney or intestine was collected from small fish (less than about 60mm) and only a portion of the lower intestine or posterior kidney was collected from larger fish. Both tissue samples were placed into the same

well of a 96-well storage plate. Each well was pre-filled with 150 $\mu$ l of a transport buffer solution (NUCPREP digestion buffer, Applied Biosystems, Foster City, CA). The 96-well plate was kept on an ice pack to keep samples and buffer cool, and after all fish were sampled the plate was transferred to dry ice and kept frozen until DNA extraction was performed.

Tissue collection for Histology – Biweekly, ten fish from each site were collected for histology. The gastrointestinal tract (pyloric ceca and intestine) and kidney were removed from the fish and immediately fixed in Davidson's fixative (Humason 1979).

### **Laboratory Assays**

Quantitative real-time PCR (QPCR)– DNA was extracted with an Applied Biosystems Model 6100 Nucleic Acid Prep Station using reagents and procedures recommended by Applied Biosystems (Foster City, CA); extracted DNA was stored at -20°C until QPCR assays were performed. QPCR utilizes a unique fluorogenic probe with a reporter molecule, and a quencher dye, commonly called the TaqMan® probe. Specific primers and probe sets are used to detect and quantify parasite DNA in real-time using a sophisticated fluorometer and specialized software (Applied Biosystems, Foster City, CA). In the QPCR assay, samples containing target DNA undergo amplification, increasing the quantity of specific DNA and associated fluorescence during each reaction cycle. Change in fluorescent signal ( $\Delta R_N$ ) was measured for each sample well and normalized against a passive background dye.

Gastrointestinal tissues were tested for *C. shasta* 18S rDNA by the method of Hallett and Bartholomew (2006). Kidney tissues were tested for *P. minibicornis* 18S rDNA utilizing Taqman Minor-Grove-Binding (MGB) probe and primers designed in Primer Express v3.0 (Kimberly True, unpublished data). The *P. minibicornis* MGB probe and primers were designed to lie within the 18S rDNA sequence published for standard PCR primers (Palenzuela et al. 1999; St.Hiliare et al. 2002). The forward primer (PmKT04-F CAAGGAGCCCGGAGCATT), Taqman MGB probe (Pm-MGB CCAACAGCATAATTGGT) and reverse primer (PmKT04-R TTACATTCAACTAAATCACACTGTTACA) produce a 92pb amplicon. A BLAST search in GenBank did not identify any other similar sequences for myxozoan or potential host species. Positive test results were reported as Cycle threshold (Ct), the point during the assay cycles when the normalized fluorescent signal surpasses background levels. Individual assay thresholds were calibrated to known positive control tissues for *C.shasta*, and *P.minibicornia*. In addition to Ct value, amplification was verified for each sample using additional features of the software which permit viewing each samples “amplification signature” and resultant change in normalized fluorescent signal ( $\Delta R_N$ ).

For *P. minibicornis*, a 30 $\mu$ L reaction mixture consisted of: 15 $\mu$ L Taqman universal master mix (Applied Biosystems, Foster City, CA), 900nM of each forward and reverse primer, 250nM Taqman® MGB Probe, molecular grade water and 5 $\mu$ l DNA template (approximately 300ng / reaction). Tests were run in an Applied Biosystems 7000 Sequence Detection System® under the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min.

Detection limits for *P. minibicornis* QPCR were determined by running extinction assays of 8 replicates of 10-fold serial dilutions of known positive tissue.

Using Poisson distribution and variance between replicates (2 standard deviations approximates a 95% confidence interval), the reliable detection limit of the *P. minibicornis* QPCR assay was determined at Ct 38. Similar extinction assays were performed for *C. shasta* QPCR, which utilizes a FAM-TAMRA fluorogenic probe rather than a MGB probe. A Ct value of 38 was used as the negative-positive cutoff for both *P. minibicornis* and *C. shasta* assays in this study.

**Histology** –After 24 hours in fixative, tissues were transferred to 70% ethanol, processed for 5 µm 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 both low (40X) and high magnification (400X). A composite infection and disease rating was developed based on the degree of tissue inflammation associated with the presence of the parasites. *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, but inflammation in <33% of the kidney section) or uninfected (no *P. minibicornis* detected).

## RESULTS

### ***Ceratomyxa shasta* detection by QPCR**

*Ceratomyxa shasta* infections were first detected in salmon collected on 6 April from the Shasta to Scott River reach (Table 2; Figure 2). The weekly prevalence of infection in the Shasta to Scott River reach (the longest data set) peaked at 100% on 11 May, then declined to 32% by 15 June, with prevalence of 57% and 48% in the last 2 weeks of sampling in this reach (Figure 2). Due to multiple rain events in May, Klamath River flows increased from 1370 cfs on 1 May to 5520 cfs on 18 May then receded to below pre-rain flow levels by the end of May (California Data Exchange Center website, <http://cdec.water.ca.gov>, Iron Gate Dam releases). The decline in incidence of *C. shasta* infections occurred during this period of increased flows. Incidence of infection in the Klamath Estuary was 10% in mid June and 13% in early July. No *C. shasta* DNA was detected in the 40 fish sampled from the lower Trinity River.

### ***Parvicapsula minibicornis* detection by QPCR**

Prevalence of *P. minibicornis* infection was 0% on 9 March (first collection) and increased each week peaking at 100% on 28 April (Table 3). The incidence of infection in fish from the Shasta to Scott River reach (our longest data set) remained high ( $\geq 83\%$ ) through 29 June (Figure 3). The infection rates remained high in fish from all sample sites in the Klamath River ( $>75\%$ ) through our last sample day on 6 July (week 18). There was no obvious change in the incidence of *P. minibicornis* infection due to the high flows in May. Incidence of infection in the Klamath Estuary was 80% in mid June and 77% in early July. *Parvicapsula minibicornis* infection was detected in 33% (13 / 40 fish) of fish collected from the Trinity River on 13 July.

## **Dual infections**

Due to the high incidence of *P. minibicornis* infection nearly all (545 / 557 = 97.8%) of the *C. shasta* infected fish were infected with both pathogens. These dual infections were observed in 38.2% (545/1428) of fish all fish examined.

## ***Ceratomyxa shasta* incidence by histology**

The weekly prevalence of *C. shasta* infection assayed by histology followed a similar trend to that seen in the QPCR assay (Figure 4). No infection was noted in salmon from the Shasta to Scott River reach during March and early April. The first parasites were seen in the gastrointestinal tract of 2 of the 30 fish sampled on 20 April. Both the prevalence of infected fish and the percentage of clinical infections peaked in mid May. The incidence of infection and percentage of clinically infected fish declined in late May and June. In addition to fish summarized in Figure 4, histology samples were also collected on 15 June in the Scott to Salmon River reach (3 of 10 fish infected with 1 clinical infection) and from the Klamath Estuary on 15 June (1 of 10 fish with clinical infection) and 6 July (none of 18 fish infected). In the 15 June sample, prevalence and severity of infection in fish from the Scott to Salmon River reach and Klamath Estuary appeared similar to fish collected in other reaches of the Klamath (10-30% infected). By early July, the rates of infection and disease in fish from the Klamath Estuary declined.

## ***Parvicapsula minibicornis* incidence by histology**

The incidence of *P. minibicornis* infection observed in kidney sections followed a similar trend to that seen in the QPCR assay (Figure 5). No infections were detected in the first few weeks of the study, and prevalence of infection increased rapidly following initial detection on 20 April to greater than 90% two weeks later. The highest incidence of clinically infected fish was observed on 18 May (14 / 15 = 93%) in the Shasta to Scott River reach. In addition to fish summarized in Figure 5, histology samples were also collected on 15 June in the Scott to Salmon River reach (10 of 10 fish infected with 9 clinical infections), 15 June in the Klamath Estuary (6 of 10 fish infected with 4 clinical infections) and 6 July in the Klamath Estuary (6 of 18 fish infected with 5 clinical infections). Prevalence and severity of infections in fish from the Scott to Salmon River reach appeared similar to fish collected in reaches upstream and downstream that same week (100% infected). Fish collected in the Klamath Estuary appeared to have lower rates and severity of infection compared to fish captured in the Klamath above the confluence of the Trinity River.

## **Histology/QPCR Correlation**

*Ceratomyxa shasta* – The assays were in agreement on 146 (66%) of the 222 fish tested by both QPCR and histology. Where the assays disagreed, 58 (26%) were positive by QPCR and undetected by histology, and 18 (8%) were positive by histology and undetected by QPCR. *Ceratomyxa shasta* trophozoites were observed in fish whose QPCR Ct score ranged from 19 (high concentration of parasite DNA) to 38 (positive/negative threshold); however, agreement was generally poor for fish with lower quantities of parasite DNA (QPCR Ct score of > 30; Figure 6). Clinical infections were

noted in histology sections from fish with QPCR Ct scores throughout the observed range; however, fish with clinical infections had a median Ct score of 27.

*Parvicapsula minibicornis* – The assays were in agreement on 175 (80%) of the 218 fish assayed by both QPCR and histology. Where the assays disagreed, 40 fish (18%) were positive by QPCR and undetected by histology, and 3 fish (1%) were positive by histology and undetected by QPCR. Infections were detected by histology primarily at QPCR Ct scores of 27 or less, and only two (7%) of the 27 infections with QPCR Ct scores of 28 or greater were detected by histology (Figure 7). Clinical infections were noted in histology sections from fish with QPCR Ct scores of 23 or less, and the median Ct score of clinically infected fish was 21.

## **DISCUSSION**

### **Comparison with Previous Studies**

In order to examine the seasonality of infections and disease in pre-smolt salmon, the sampling in 2005 began weeks earlier than any previous fish health surveys on the Klamath River. In a review of *C. shasta* literature, Bartholomew et al. (1989) cite infections occurring at water temperatures as low as 4 to 6°C with disease progressing faster at warmer water temperatures. The water temperatures at which infections were first detected in this study (8 °C for *P. minibicornis* and 11 °C for *C. shasta*) were well above these minimum published temperatures. Juvenile Klamath River Fall Chinook Salmon historically began out-migration in February, peak in mid-June, and are captured in Klamath Estuary in large numbers in June through mid August (Leidy and Leidy 1984; Wallace and Collins 1997). For interannual comparisons of parasite infection, we limited the 2005 data set to the months of May, June and July. These months bracketed the typical peak of Fall Chinook out-migration and included the pathogen monitoring periods from previous years. The *C. shasta* incidence of infection observed during May-July, 2005 (62% by QPCR and 35% by histology) was similar to that observed in previous studies (34% by histology in 2004 and 0%-50% by histology in 1994-2002). The *P. minibicornis* infection during May-July, 2005 (92% by histology and 96% by QPCR) was at the upper range seen in past years (77% by histology in 2004, and 47%-88% by histology in 1995-2002).

### **Incidence of infection differs between reaches**

Weekly infection rate in fish from one reach was not a good predictor of infections in other reaches. Differences were noted between fish from the Shasta to Scott River reach and Salmon to Trinity River reach, and between fish from reaches above the confluence of the Trinity River and the Klamath Estuary. Differences between reaches have several possible explanations. Tributary fish would typically be uninfected upon entering the mainstem Klamath and a “pulse” of fish from a tributary could significantly reduce the incidence of infection in a sample. It took fish some time to migrate down the river and there may have been a delay of weeks between sampling a group of fish in an upstream reach and again in a reach further downstream. Infected fish may die during out-migration reducing the incidence of infection in downstream reaches such as the estuary. Previous studies have shown that following a high dose exposure ceratomyxosis was detectable within days post-infection (by histology) with peak mortality occurring

about 2 weeks post infection at 16 to 20°C water temperatures (Foott et al. 2004). Given that fish continually become infected as they migrate through or rear in the Klamath River, there was no reason to expect the incidence of infection in reaches or sample sites with significant distances between them to correlate.

### **Comparison of histology with QPCR**

The sensitivity of QPCR was clearly higher than that achieved with histology. This difference was more apparent in the assay for *P. minibicornis* than *C. shasta*. The QPCR assay was able to reliably detect infections at parasite levels far below histological detection (Hallett and Bartholomew 2006). Histological examination of infected fish allowed us to rate the severity of tissue damage, an important characteristic when considering the prognosis of the infection. The relatively poor correlation of *C. shasta* histology and QPCR assay was likely due to the focal nature of *C. shasta* within the intestine. The limits on the amount of tissue in the QPCR assay (requiring a sub-sample of the intestine) and the nature of the histology assay (5 µm thick section) led to false negative results in both assays. In the QPCR assay at least, this limitation may be overcome in the future by homogenizing the entire intestine and assaying a subsample of this homogenate. The histology assay for *C. shasta* will continue to face a challenge when attempting to diagnose early or subclinical infections due to the focal nature of the infection.

### **Seasonality of the parasites**

Incidence and severity of infection for both *C. shasta* and *P. minibicornis* tended to increase throughout the spring and may be associated with water temperature. Peak incidence occurred prior to the bulk of Chinook salmon out-migration, while the fish were relatively small (mean fork length 57-67mm). Seasonality of the infection during the spring of 2005 may not be typical due to the unusually heavy storm in May which significantly increased flows and reduced water temperature through the month of May (Figures 2 and 3).

### **Differences in *C. shasta* and *P. minibicornis* infection patterns**

There was a decrease in incidence of *C. shasta* infection which coincided with increased flows during the month of May. The decrease in *C. shasta* incidence was possibly due to a pulse of uninfected fish entering the system. Iron Gate Hatchery released 5.3 million juvenile Chinook between 17 May and 3 June, and uninfected naturally produced fish may also have moved out of tributaries with the increased flows. The incidence of *P. minibicornis*, however, remained high following the storm and through the end of the study. Hatchery fish had subclinical *P. minibicornis* infections prior to release (J, Bartholomew, personal communication, 28 August, 2006) which may explain the difference in *C. shasta* and *P. minibicornis* infection patterns. Another factor affecting the pattern of infection was differences in concentrations of *C. shasta* and *P. minibicornis* actinospores (infectious stage) in the water. Studies looking at fish infection rates and parasite concentrations in the water both conclude the concentration of *P. minibicornis* was higher than *C. shasta* in the Klamath River during the spring 2005 (Foott et al. 2006). It was also possible that *C. shasta*-infected fish die at a higher rate

than *P. minibicornis*-infected fish. This increased mortality rate would remove *C. shasta* infected fish from the population.

### **Prognosis of *C. shasta* and *P. minibicornis* infections**

The incidence of *C. shasta* infection decreased in the month of May (discussed above), but the incidence of infection for both *C. shasta* and *P. minibicornis* reached 100% prior to this drop. Hatchery fish were not released until mid May, so fish sampled during this 100% infection period were naturally produced. The more sensitive QPCR assay in this study allows detection of lower level infections which may not always lead to disease and mortality. In past studies using the less sensitive histology detection technique, low survival was expected from *C. shasta* infected fish. Sentinel salmon exposed in the Shasta to Scott River reach resulted in 82% mortality in less than 3 weeks at 16°C and warmer water temperatures decrease mean survival time (Udey et al. 1975, Foott et al. 2004). The progress of *P. minibicornis* infection in juvenile Chinook salmon is not well understood and this is an important question given the high prevalence of infection (77-100%) observed in May, June and July of 2005. The high prevalence of *P. minibicornis* infections results in nearly all (98%) of the *C. shasta* infected fish having dual infections. We hypothesize that the probability of infection for both pathogens was related to the duration of residence within the mainstem Klamath River, with natural Chinook juveniles infected as early as March and April. A useful next step would be to model disease progression as a function of residence time in the mainstem Klamath River. Large numbers of marked fish would be required such a study.

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Table 1. Sample sites and sample reaches in the Klamath River (KR) from Iron Gate Dam (IGD) to the estuary and Trinity River (TR). Sites were sampled by rotary screw trap (T), beach seine (S) or electrofishing (E).

<b>Reach Description</b>	<b>River Miles</b>	<b>Sites</b>
IGD to Shasta R.	KR 187-177	Bogus (T), Interstate-5 (T), Cottonwood (S), Copco/Ager (S)
Shasta R. to Scott R.	KR 177-143	Tree of Heaven (S & E), Kinsman (T)
Scott R. to Salmon R.	KR 143-66	Happy Camp (S), Persido Bar (S)
Salmon R. to Trinity R.	KR 66-44	Big Bar (T & S)
Trinity R. to Estuary	KR 44-3	No sites sampled
Klamath Estuary	KR 0-3	Klamath Estuary (S)
Trinity River	TR 21	Willow Creek (T)

Table 2. Weekly prevalence of *Ceratomyxa shasta* infection by QPCR in juvenile Chinook salmon sampled from 5 reaches within the Klamath River and lower Trinity River. The prevalence (#positive/#sampled) is presented for each sample reach by collection week and sample date.

Collection Week	Sample Date	Iron Gate Dam to Shasta R.	Shasta R. to Scott R.	Scott R. to Salmon R.	Salmon R. to Trinity R.	Klamath Estuary	Lower Trinity R.
1	9-Mar	0% (0/30)	0% (0/30)				
2	16-Mar	0% (0/23)	0% (0/50)				
3	23-Mar		0% (0/37)				
4	30-Mar	0% (0/30)	0% (0/56)				
5	6-Apr	0% (0/26)	15% (9/60)				
6	13-Apr	0% (0/25)	23% (8/35)				
7	20-Apr	7% (2/28)	37% (21/57)				
8	28-Apr	77% (23/30)	65% (37/57)				
9	4-May	43% (3/7)	73% (44/60)				
10	11-May	100% (30/30)	100% (39/39)				
11	18-May	60% (15/25)	80% (28/35)		88% (22/25)		
12	25-May		63% (38/60)		92% (24/26)		
13	2-Jun		47% (25/53)		70% (21/30)		
14	8-Jun		47% (14/30)	82% (9/11)			
15	15-Jun		32% (8/25)	24% (6/25)	50% (11/22)	10% (1/10)	
16	22-Jun		57% (25/44)		45% (20/44)		
17	29-Jun		48% (21/44)		24% (11/45)		
18	6-Jul				75% (33/44)	13% (11/88)	
19	13-Jul						0% (0/40)

Table 3. Weekly prevalence of *Parvicapsula minibicornis* infection by QPCR in juvenile Chinook salmon sampled from 5 reaches within the Klamath River and lower Trinity River. The prevalence (#positive/#sampled) is presented for each reach by collection week and sample date.

Collection Week	Sample Date	Iron Gate Dam to Shasta R.	Shasta R. to Scott R.	Scott R. to Salmon R.	Salmon R. to Trinity R.	Klamath Estuary	Lower Trinity R.
1	9-Mar	0% (0/30)	0% (0/30)				
2	16-Mar	9% (2/23)	2% (1/50)				
3	23-Mar		3% (1/37)				
4	30-Mar	0% (0/30)	21% (12/56)				
5	6-Apr	35% (9/26)	78% (47/60)				
6	13-Apr	72% (18/25)	69% (24/35)				
7	20-Apr	93% (26/28)	79% (46/58)				
8	28-Apr	100% (30/30)	100% (57/57)				
9	4-May	100% (7/7)	98% (58/59)				
10	11-May	97% (29/30)	90% (35/39)				
11	18-May	96% (24/25)	100% (35/35)		84% (21/25)		
12	25-May		97% (58/60)		96% (24/25)		
13	2-Jun		100% (53/53)		100% (30/30)		
14	8-Jun		83% (25/30)	100% (11/11)			
15	15-Jun		100% (25/25)	100% (25/25)	100% (22/22)	80% (8/10)	
16	22-Jun		89% (39/44)		95% (36/38)		
17	29-Jun		100% (44/44)		100% (45/45)		
18	6-Jul				100% (44/44)	77% (68/88)	
19	13-Jul						33% (13/40)

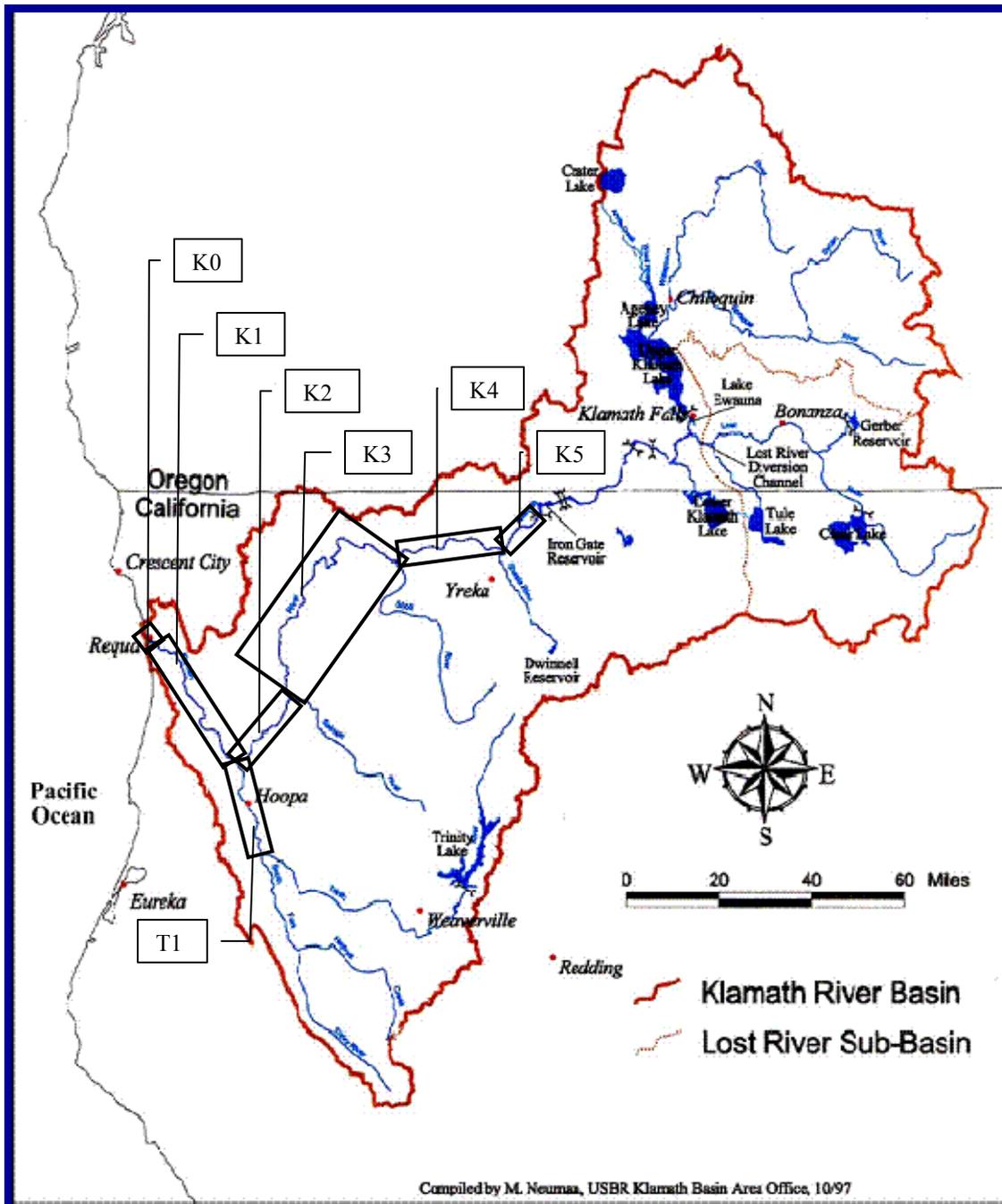


Figure 1. Map of sample reaches in the Klamath River Basin. Reaches are identified as K0 – Klamath Estuary, K1 – Estuary to Trinity River, K2 – Trinity River to Salmon River, K3 – Salmon River to Scott River, K4 – Scott River to Shasta River, K5 – Shasta River to Iron Gate Dam, T1 – Trinity River from confluence to South Fork Trinity River

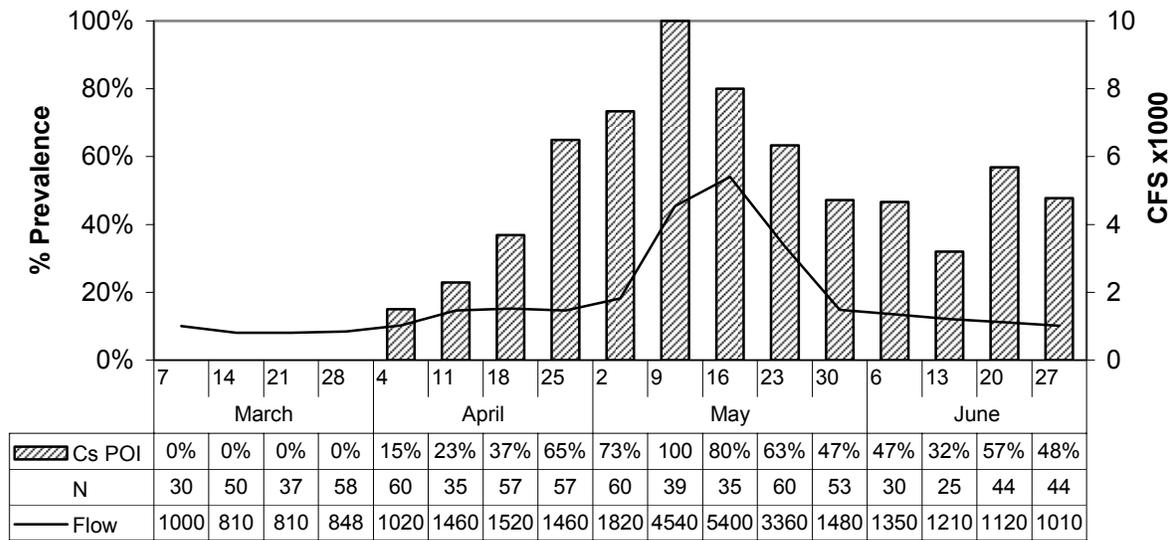


Figure 2. Weekly prevalence of *Ceratomyxa shasta* infection (Cs POI) in juvenile Klamath River Chinook salmon collected in the Shasta to Scott River reach. N is the total number of fish assayed for a given sample week. Calendar dates are the Monday of each sample week. Flow (CFS) is average weekly Iron Gate Dam releases.

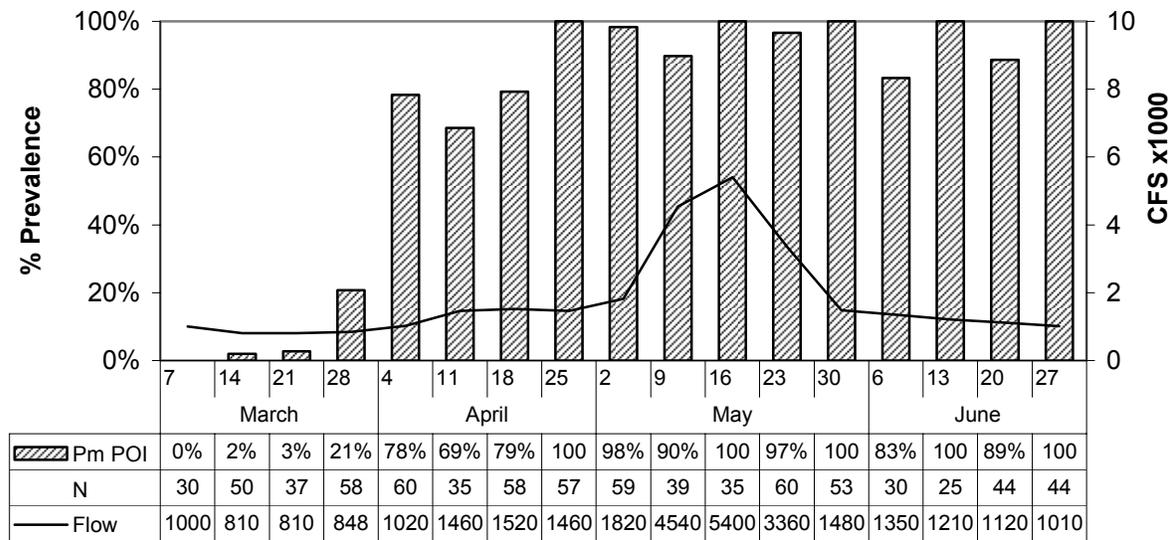


Figure 3. Weekly prevalence of *Parvicapsula minibicornis* infection (Pm POI) in juvenile Klamath River Chinook salmon collected in the Shasta to Scott River reach. N is the total number of fish captured in a given collection week. Calendar dates are the Monday of each sample week. Flow is average weekly Iron Gate Dam releases.

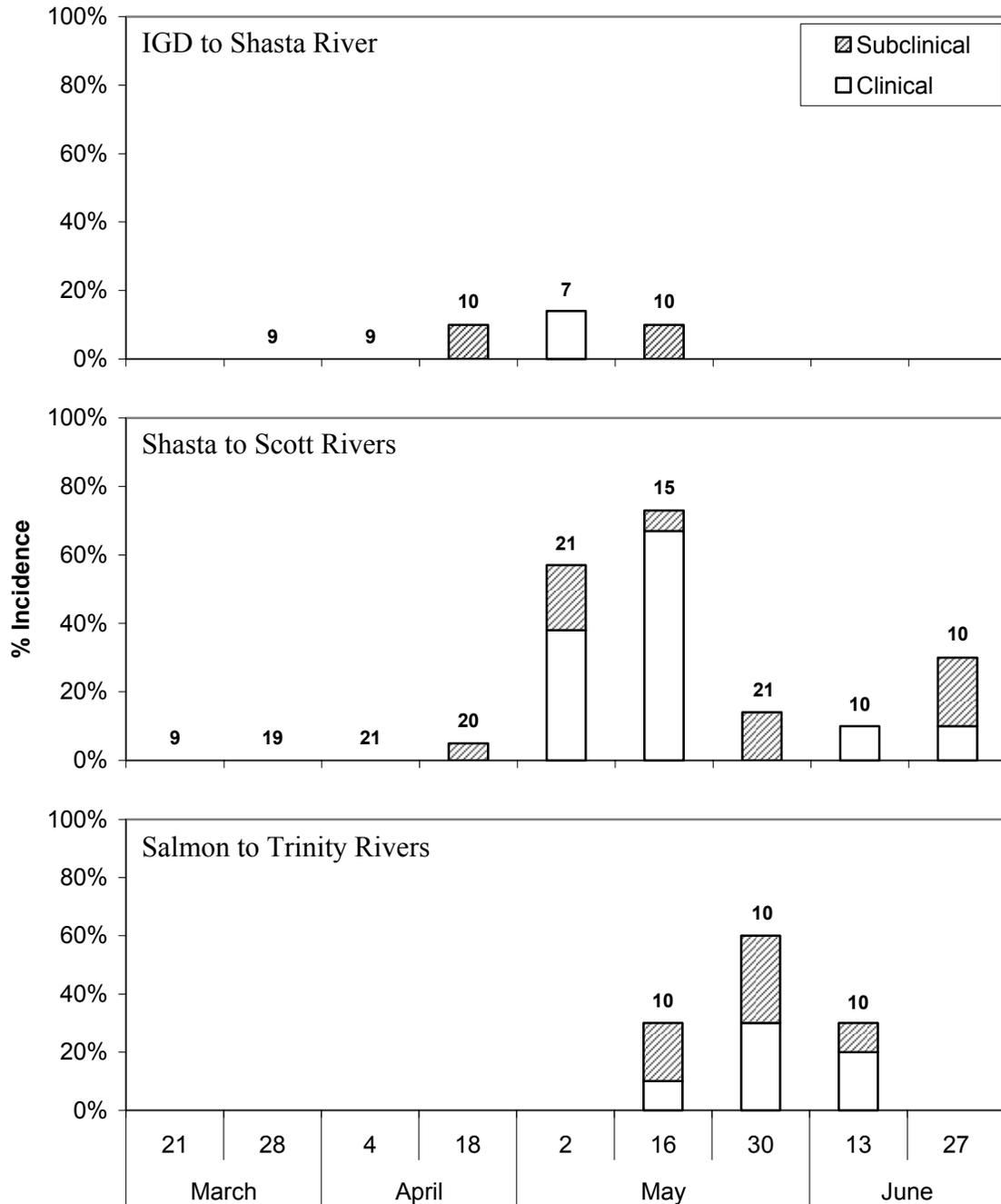


Figure 4. Incidence and severity of *Ceratomyxa shasta* infection detected by histological assay of intestine sections from Klamath River Chinook salmon collected in the spring and summer of 2005. All fish collected within a sample reach (Iron Gate Dam to Shasta River, Shasta River to Scott River, or Salmon River to Trinity River) in a given week were combined. Calendar dates are the Monday of each sample week. Data presented as percent of fish which had light or early infections (Subclinical) and percent of fish infected with associated inflammation in more than 33% of the intestinal section (Clinical). Number of samples (n) examined for each week is given at the top of each bar.

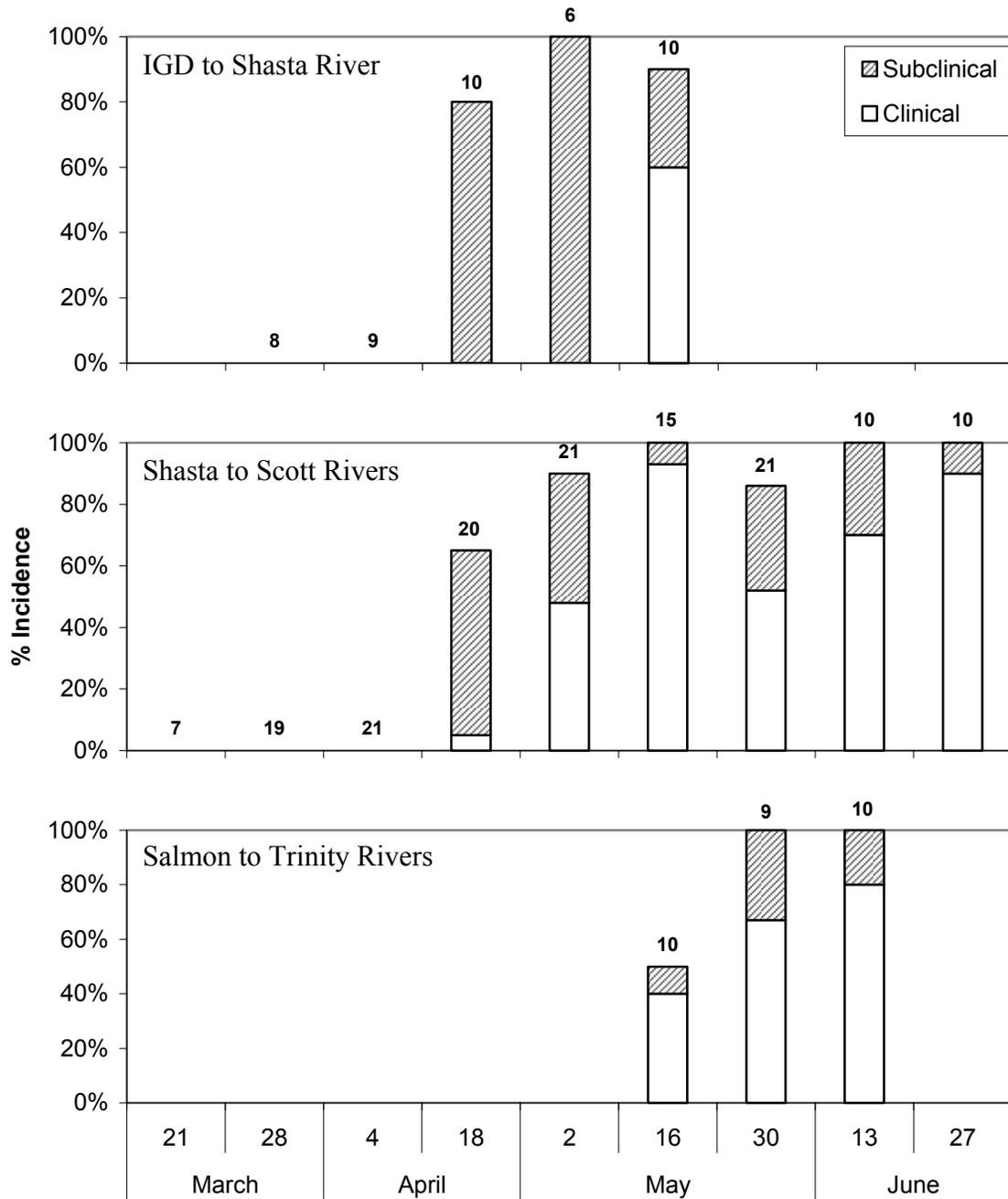


Figure 5. Incidence and severity of *Parvicapsula minibicornis* infection detected by histological assay of kidney sections from Klamath River Chinook salmon collected in the spring and summer of 2005. All fish collected within a sample reach (Iron Gate Dam to Shasta River, Shasta River to Scott River, or Salmon River to Trinity River) in a given week were combined. Calendar dates are the Monday of each sample week. Data presented as percent of fish which had light or early infections (Subclinical) and percent of fish infected with associated glomerulonephritis in more than 33% of the kidney section (Clinical). Number of samples (n) examined for each week is given at the top of each bar.

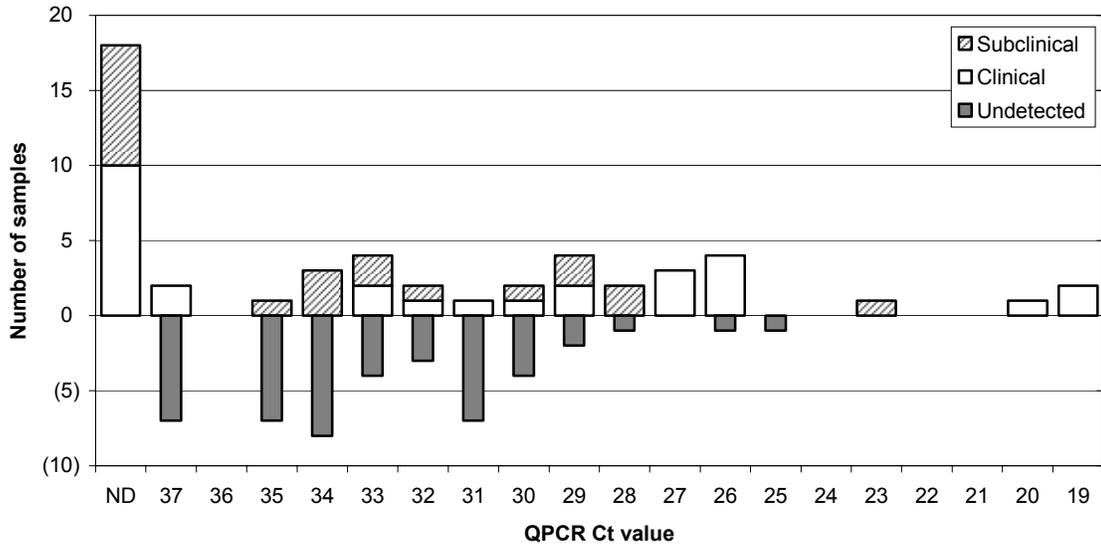


Figure 6. Relationship between QPCR Ct value and infection rating by histology for fish examined by both methods for *Ceratomyxa shasta*. Parenthetic numbers were detected only by QPCR. Histological data presented as number of samples with light or early infections (Subclinical) plus samples with associated inflammation in more than 33% of the intestinal section (Clinical). A QPCR Ct value of “ND” indicates the parasite was not detected by the QPCR assay. Fish which were negative by both methods (114 fish) were not shown.

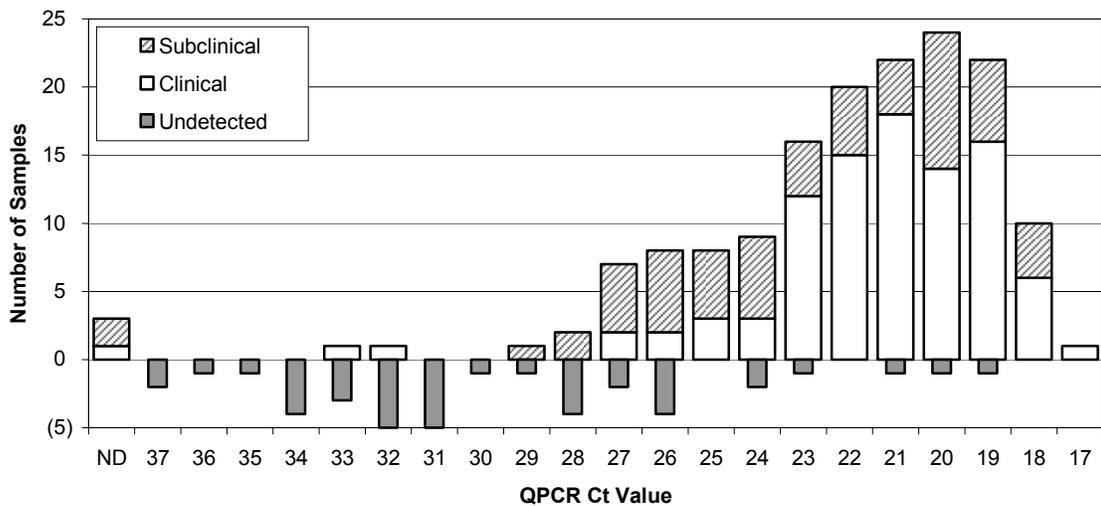


Figure 7. Relationship between QPCR Ct value and infection rating by histology for fish examined by both methods for *Parvicapsula minibicornis*. Parenthetic numbers were detected only by QPCR. Histological data presented as number of samples with light or early infections (Subclinical) plus samples with associated glomerulonephritis in more than 33% of the kidney section (Clinical). A QPCR Ct value of “ND” indicates the parasite was not detected by the QPCR assay. Fish which were negative by both methods (23 fish) were not shown.

## **Appendix I. Reviewers' Comments on Draft Report**

This is just a suggestion to clarify that it was a real-time (quantitative) assay in that paper rather than the standard PCR.

Was the cut-off for *C. shasta* the same [as that described for *P. minibicornis*]?

Have you taken into account that fish coming from IGH may already be infected with Pm? You may be starting with a higher baseline Pm infection in the general out-migrant population.

Any evidence that these fish might have spent time in the Klamath and then gone back up into the Trinity? We have detected a very low infection prevalence in sentinel fish held in the Trinity, but not high enough to suggest this level.