U.S. Fish & Wildlife Service

California Nevada Fish Health Center
FY2006 Investigational Report:

Comparative susceptibility to infection and disease from *Ceratomyxa shasta* and *Parvicapsula minibicornis* in Klamath River basin juvenile Chinook, Coho and Steelhead populations.

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Summary: Six stocks of juvenile Klamath River basin salmonids (Salmon River Chinook salmon, Shasta River Chinook salmon, Iron Gate Hatchery Chinook salmon, Iron Gate Hatchery coho salmon, Trinity River Hatchery Chinook salmon and Trinity River Hatchery steelhead) were exposed for 72 h in the Klamath River during June 2006, held for an additional 16 d in 19°C parasite-free water and histologically evaluated for both incidence of infection (measure of susceptibility) and severity rating of each infection (measure of disease progression) by *Ceratomyxa shasta and Parvicapsula minibicornis.* All Chinook stocks had similar high levels of infection and disease by both parasites. Iron Gate Hatchery coho appear to be highly susceptible to infection by both parasites however they showed a slower progression to clinical disease (ceratomyxosis) than the Chinook groups. Trinity River Hatchery steelhead had a high incidence of *P. minibicornis* infection but demonstrated an innate resistance to infection by *C. shasta.*

Introduction:

Juvenile salmon in the Klamath River incur a high incidence of infection of two myxozoan parasites, *Ceratomyxa shasta* and *Parvicapsula minibicornis* (Stocking et al. 2006, Foott et al. 2004). These parasites occur in a number of Pacific Northwest watersheds and the life cycles of both parasites include the polychaete, *Manayunkia speciosa,* as an alternatehost (Bartholomew et al. 1997, Bartholomew et al. 2006). The actinospore, a stage that is infectious to salmon, is released from infected polychaetes into the water column. Infections by *C.shasta* can occur from spring through fall at water temperatures $\geq$ 7°C (Ching & Munday 1984, Hendrickson et al. 1989). Seasonal infectivity data for *P. minibicornis* has not been reported but appears to be similar to *C. shasta* in the Klamath River (Nichols and True, 2007). It is important to differentiate between the term infection and disease. In the context of myxozoan infection of juvenile Klamath R. salmonids, disease progression acts to compromise the fitness of the fish and reduce its survival. Infection is defined in Dorland's Medical Dictionary (1982): "as invasion and multiplication of microorganisms in the body tissues, especially that causing local cellular injury due to competitive metabolism, toxins, intracellular replication, or antigen-antibody response". Similarly disease is defined as: "deviation from or interruption of the normal structure or function of any body part, organ, or system that is manifested by a characteristic set of symptoms and signs and whose etiology, pathology, and prognosis may be known or unknown". Our primary focus in this study was to determine differences in susceptibility to infection and disease progression by *C. shasta* and *P. minibicornis* among Klamath River basin Chinook salmon *(Oncorhynchus tshawytscha)*, coho salmon *(O. kisutch)* and steelhead trout *(Oncorhynchus mykiss).* We used a histological scoring system to differentiate the progression of parasitic infections into various levels of disease.

Methods:

Fish- Juvenile (0+) Klamath River stock Chinook and coho salmon were obtained from the California Department of Fish and Game (CDFG) Iron Gate

Hatchery (IGH) 20 days (d) prior to the June 14, 2006 exposure date. Other juvenile Chinook salmon and steelhead trout were obtained from CDFG Trinity River Hatchery (TRH) 5 d prior to the exposure date. Two stocks of naturally produced juvenile Chinook salmon were also used in the experiment. Shasta River salmon were obtained from a CDFG screw trap near the river mouth on two separate dates. The first group was obtained 20 d prior to the exposure while the second group was obtained on the morning of June 14th and trucked directly to the exposure site. Juvenile Salmon River Chinook were obtained by the Karuk Tribal Fisheries Program (rotary screw trap) 20 d prior to exposure. Due to extremely low juvenile Chinook abundance in the Scott river, we were unable to obtain fish from this river (Bill Chesney, CDFG, personal communication). Final numbers of each salmonid population used in the study are listed in Table 1.

Fish obtained prior to the exposure date were brought back to the California Nevada Fish Health Center wet laboratory (with the exception of the above mentioned Shasta R. group) and held in separate 40 L aquaria supplied with 18.9 L min^{-1} flow of single-pass, ozone- treated water at temperatures similar to the Klamath River (mean daily temperature of 19.1º C, range = 16.6 – 20.9 ºC). The lab receives Coleman National Fish Hatchery water and there is no history of either *C. shasta* or *P. minibicornis* infection at this facility.

In order to reduce the occurrence of columnaris disease (infection by *Flavobacterium columnare*), 10 minute prophylactic baths of 1 mg L^{-1} furanase were administered for two consecutive days post arrival. Hatchery fish also received oxytetracycline medicated feed (7g active / 100 lbs of fish) for a week after exposure. These medications do not affect infection by myxozoan parasites. Soon after capture, wild Chinook from the Salmon and Shasta rivers were observed to be infected with columnaris and required additional furanase treatments. Hatchery produced salmon were fed a 1.0mm Silvercup salmon diet while wild fish were fed live tubifex worms. Effluent from the wet lab was chlorinated.

Exposure- Eleven cages of sentinel fish were transported to the Klamath River the morning of June 14, 2006. Actual number of fish exposed per group can be found in Table 1. The exposure site was approximately 1 river mile above the mouth of Beaver Creek (see title page photograph, rkm 262, UTM 10 516058E 4634926N). The 1.58 ft long cages were constructed of 6 inch PVC pipe with multiple 3” holes cut into the sides for flow (chamber radius = 0.26 ft). One quarter inch mesh screen surrounded the outside of each cage. Total volume of each cage was 0.34 cf. ft. Live boxes were anchored on the bottom (~3ft depth) perpendicular to the river edge and orientated to the current so as to maximize laminar flow through the cages. After 72 h, the caged groups were transported back to the wet laboratory for the 16 d holding period as described above for a total post exposure time of 19 d . Fish were treated with 1 mg L^{-1} furanase during the last 45 min. of transport.

At 19 days post exposure (dpe) all surviving fish were sampled for histological examination (Table 1). Intestinal tract, including the pyloric caeca, and kidney were placed in Davidson’s fixative for 24 h, processed for 5 μm paraffin sections and stained with hematoxylin and eosin (Humason 1979).

Previous work has shown these post-exposure periods to produce clinical infections dominated by trophozoite stages (Foott et al. 2004). All tissues for a given fish were placed on a single slide and identified by a unique code number. Each slide was examined at low (40X) and high magnification (400X) without knowledge of the sample group. A small subset of histological specimens did not contain intestinal tract or kidney tissue of sufficient quantity or morphological quality to examine. Parasite infection was rated in the sections as **Uninfected (#0)** = no parasites seen, **Infected (#1)** = parasite present but little to no tissue damage or inflammation, **Diseased (#2)** = parasite present with focal areas of inflammation observed in less than 30% of the affected tissue and, **Diseased (#3)** = parasite present and fish had progressed to a diseased state with inflammation seen in > 30% of the affected tissue. Histology scores for each exposure group were compiled into 3 ratings (0, 1, and "diseased" {2 or 3}). We examined differences in the frequency of each score category using a Fisher Exact (2 Tail) Test due the small sample sizes (significance level of $P<0.05$)

Results:

***Early Mortality Complications*:**

Following the 72 h exposure, infection by *F. columnare* proved extremely difficult to control in 3 Chinook groups. The Salmon R., Shasta R. and IGH Chinook salmon incurred 53% mortality between 4-10 dpe that was associated with columnaris lesions (Table 1). While *C. shasta* trophozoites were observed in 47% of these early columnaris mortalities, it was difficult to observe single trophozoites in post-mortem tissues. The first disease severity rating of 2 (parasite present with focal areas of inflammation observed in less than 30% of the affected tissue) was observed at 11 dpe in both Shasta R and IGH mortalities. During this early post-exposure period, TRH Chinook had only 2 mortalities with a *C. shasta* detection in a10 dpe mortality. Both coho and steelhead had relatively few mortalities prior to 19 dpe (Table 1).

In order to increase our confidence in comparing infections among the Chinook exposure groups, fish that died between 4-10 dpe were excluded from the incidence of infection data set. Our rationale for setting 11 dpe as a cutoff point was as follows:

1. Our inclination to use the same data set for both incidence of infection and disease (first disease rating occurring at 11 dpe).
2. The low mortality that occurred in the TRH Chinook prior to 11 dpe would have weighed their disease ratings towards individuals that had a longer time for disease progression than the other 3 Chinook groups.
3. Lower confidence in observing trophozoites in post-mortem intestines could have biased incidence of infection towards fish sampled after 10 dpe.

A subset of the mortalities occurring between 11 and 19 dpe (those showing limited post-mortem changes) were sampled for histological examination and incorporated into the data set. We were unable to assign *C.shasta*-specific mortality curves for the Chinook groups due to complications with *F. columnaris* infections.

Table 1. Sentinel composition and mortality data for exposure groups (Salmon River Chinook, Shasta River Chinook, Iron Gate Hatchery Chinook (IGH), Trinity River Hatchery (TRH) Chinook, IGH coho and TRH steelhead. Data reported as: number of fish retained in the laboratory and later evaluated as controls, sentinel fish exposed in the Klamath R., mortalities (Morts.) over the 72 h exposure (Exp.), mortalities 4-10 days post exposure (DPE), mortalities 11 -18 DPE, fish sampled at 19 DPE, intestine (INT) and kidney (KD) sections examined per exposure group.

Sentinel Groups	Control Fish	Exposed Fish	Morts. Exp.	Morts. 4-9 DPE	Morts. 10-18 DPE	Sample 19 DPE	**INT**	**KD**
Salmon R Chinook	3	29	1	18	4	6	**6**	**6**
Shasta R. Chinook	24	56	16	29	11	0	**8**	**8**
IGH Chinook	25	58	0	25	19	14	**14**	**15**
TRH Chinook	24	69	0	17	33	19	**26**	**26**
IGH Coho	14	49	0	1	0	43**	**43**	**40**
TRH Steelhead	16	48	9	4	2	33	**33**	**33**

** Four mortalities recovered on 19 dpe were too necrotic for histological examination and one section was lost.

***Ceratomyxa shasta*:**

Small sample size in naturally produced fish groups precluded the use of statistical analysis between all four Chinook groups (Table 1). There was no statistical difference observed in the incidence of infection between Shasta R. and Salmon R. groups (P = 0.538) or between the two hatchery groups (P = 0.416). All Chinook groups appear to be highly susceptible to *C.shasta* infection (67 – 88% incidence of infection). The first observation of *C.shasta* trophozoites was in a Salmon R mortality collected at 7 dpe. Shasta R. Chinook yielded the highest incidence of infection at 88% (7 of 8 fish) followed by TRH fish at 85% (22 of 26 fish) by the end of the 19 day holding period (Fig. 1).

Both hatchery and naturally produced Chinook progressed into a disease state (histology scores $\geq$2) over the 19 day holding period (Table 2). There was no statistical difference observed in the incidence of disease between Shasta R.

and Salmon R. groups (P = 0.245) or between the two hatchery groups (P = 0.469). . A disease rating was given to 50% (3 of 6) of Salmon R. Chinook, 88% (7 of 8) of Shasta R. Chinook, 77% (20 of 26) of TRH Chinook and 64% (9 of 14) IGH Chinook (Fig 1 and Table 2).

TRH Steelhead responded differently to *C. shasta* than the Chinook exposure groups. Only 6 mortalities occurred over the 19 d study with 15% of the group (5 of 33) rated as clinically diseased (Table 1 and Fig 1). Numerous small trophozoites were observed in the intestinal lumen of 79% of the 19 dpe Steelhead intestinal sections. These "luminal" forms did not penetrate the epithelial cells (Fig. 1 and 2, Table 2).

Figure 1. Incidence of *C. shasta* infection (histology score = 1) and clinical disease (histology score of 2 or 3) in Salmon River Chinook salmon, Shasta River Chinook salmon, Iron Gate Hatchery (IGH) Chinook salmon Trinity River Hatchery (TRH) Chinook salmon, IGH coho salmon and TRH steelhead sampled 11-19 days post exposure. An additional category for non-invasive luminal (L-form) trophozoites is recorded for TRH Steelhead. Sample number (N) for each group is listed above the bars.

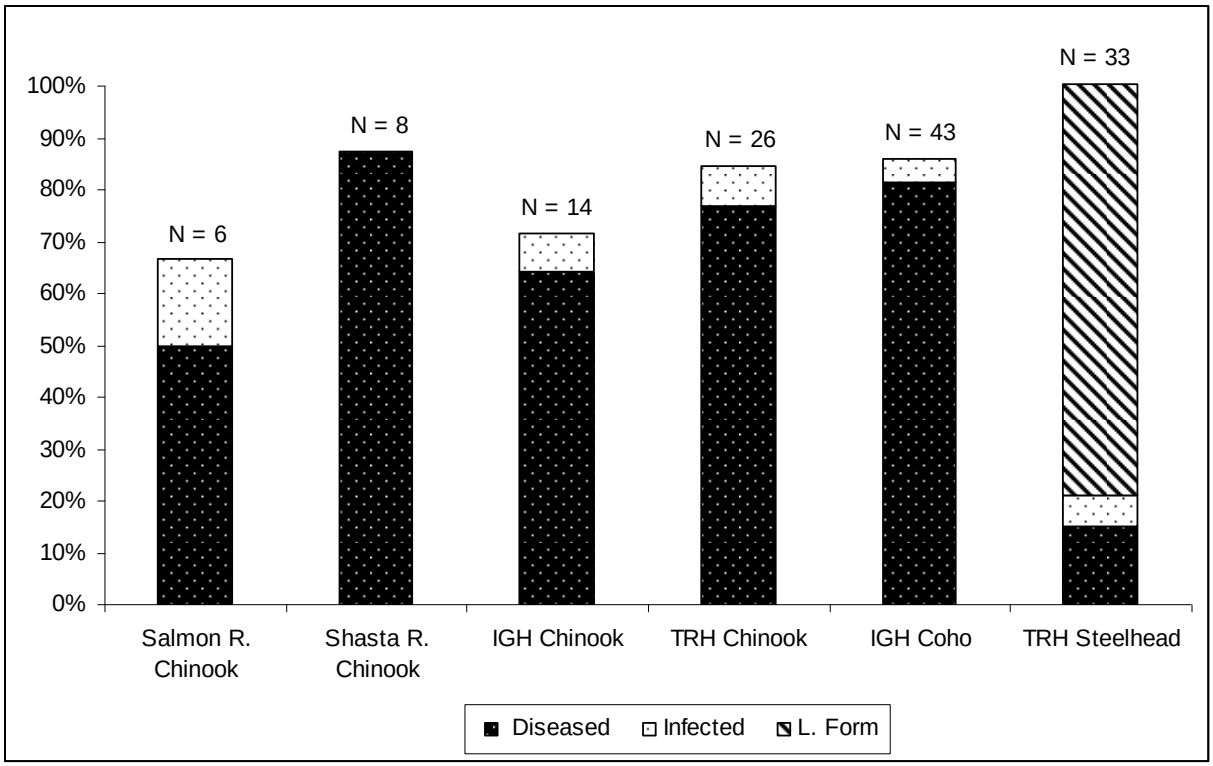
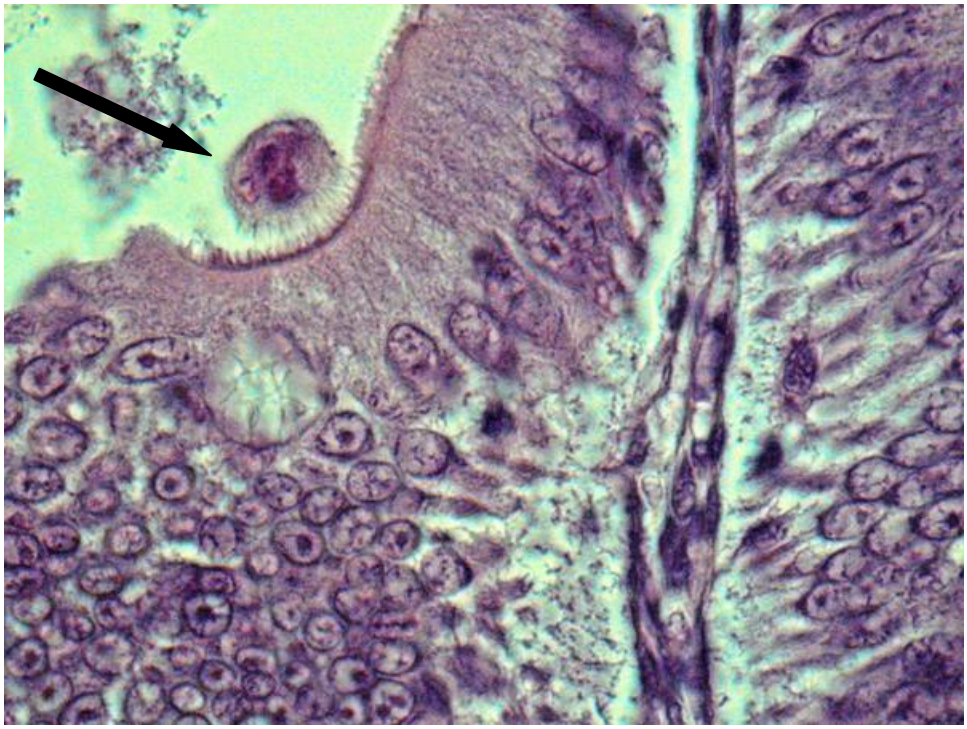


Table 2. Histological scores for *C.shasta* and *P. minibicornis* infection (0 or 1 - 3) and disease ratings (2 or 3). Reported as number of Chinook, coho, and steelhead sampled between 11 and 19 dpe for each rating. The (L) rating for steelhead *C shasta* infections refers to non-invasive luminal trophozoites and is not applicable (NA) for the other groups.

	C. shasta						*P. minibicornis*			
	0	1	*2*	3	L		0	1	2	3
Salmon R. Chinook	2	1	2	1	NA		2	1	0	3
Shasta R. Chinook	1	0	6	1	NA		0	2	5	1
IGH Chinook	4	1	3	6	NA		0	1	12	2
TRH Chinook	4	2	5	15	NA		0	0	20	6
IGH coho	6	2	10	25	NA		3	36	1	0
TRH steelhead	0	2	3	2	26		0	33	0	0

Figure 2. Photomicrograph of a presumptive *C. shasta* trophozoite (arrow) within the intestinal lumen of a TRH Steelhead sampled at 19 dpe. Invasion of intestinal epithelium has not occurred.

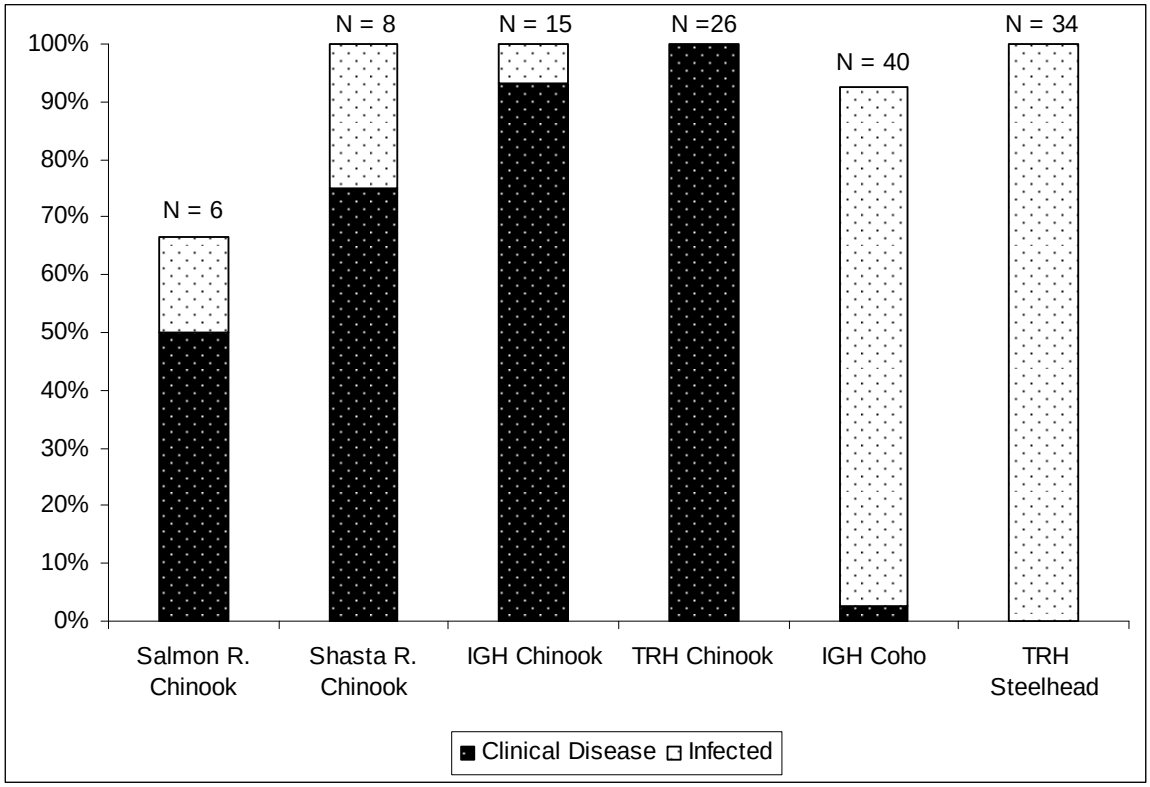


Coho salmon appeared to have similar susceptibility to infection as Chinook but progressed to clinical disease at a slower rate. Coho experienced only 10% mortality however 81% (35 of 43) of the fish sampled at 19 dpe were rated as clinically diseased. No *C. shasta* was observed in any control fish.

Parvicapsula minibicornis:

All Chinook groups had a high incidence of glomerulonephritis (50 – 88% histology score $\geq$ 2) associated with *P. minibicornis* infection. Small sample size of naturally produced groups precluded the use of statistical analysis between all four Chinook groups (Table 1 and 2). Analysis between the Shasta River and Salmon River Chinook salmon revealed no difference in their high incidence of infection (P = 0.165) or disease (P = 0.580). Both IGH Chinook and TRH Chinook groups had a 100% infection and no statistical difference (P = 0.366) was detected in their incidence of glomerulonephritis (Fig. 3). The first observation of the parasite was in a Shasta R. Chinook mortality collected at 4 dpe. TRH Steelhead had a 100% incidence of infection however no glomerulonephritis was observed in their kidneys. Similarly, IGH Coho had a high incidence of infection (93%) with only one fish rated as diseased. No *P. minibicornis* was observed in any control fish.

Figure 3. Incidence of *P. minibicornis* infection (histology score = 1) and glomerulonephritis (clinical disease, histology score = 2 or 3) in Salmon River Chinook salmon, Shasta River Chinook salmon, Iron Gate Hatchery (IGH) Chinook salmon, Trinity River Hatchery (TRH) Chinook salmon, IGH coho salmon and TRH steelhead sampled 11-19 dpe.



Discussion:

Iron Gate Hatchery coho appear to have similar susceptibility to *C.shasta* infection (86% incidence of infection) as the four Chinook stocks (67 – 88%) but showed a slower disease progression. Despite their low cumulative 19 d mortality, 81% of the exposed coho had progressed to a clinical state of disease and mortality would likely soon occur had the study been extended. Udey et al. (1975) described a similar susceptibility to ceratomyxosis in challenged coho reared at $\geq$ 20.5°C. Unlike *C.shasta*, the 93% incidence of *P. minibicornis* infection in IGH coho did not progress to clinical disease. This data indicates either a different *P. minibicornis* strain infecting coho or a different host response to infection in comparison to Chinook salmon. We have detected *C. shasta* DNA by QPCR in both 0+ and 1+ coho salmon collected in the Klamath R. (2007 preliminary data – unpublished). Ceratomyxosis should be viewed as one factor affecting coho survival in the Klamath River.

TRH steelhead demonstrated an innate resistance to *C. shasta* infection and disease progression under the limited 3d challenge of this study. Similar findings with IGH steelhead were reported by Foott et al. (2004). In a majority of the steelhead sampled, we observed large numbers of small (presumptive) *C. shasta* trophozoites in the intestinal lumen that had not penetrated the epithelial layer. Bartholomew et al. (2004) describe a similar observation in a resistant strain of steelhead exposed to infectious Cowlitz River water. Due to the inability of the parasite to penetrate the epithelial layer, we did not consider these fish to be "infected". The new category of luminal form was incorporated into our histology scoring system to account for these trout. An inheritance of susceptibility to ceratomyxosis has been reported in coho salmon and Rainbow trout with parasite dose a significant factor in resistance (Hemmingsen et al. 1986, Ibarra et al. 1992). Nichols et al. (2003) described this resistance to be polygenic. It is likely that resistance is working at several levels such as preventing initial penetration of the epithelium as well as later immune responses to any invading parasites. The latter mechanism(s) is portrayed by the intense granulomatous response observed around individual trophozoites in asymptomatic Chinook salmon exposed to limited quantities of infectious Klamath River water (Foott et al. 2007a).

Both naturally produced and hatchery stocks of Chinook salmon were essentially equivalent in their susceptibility to infection and disease by *C. shasta* and *P. minibicornis.* The extensive mortality, in 3 of 4 Chinook groups prior to 10 dpe, limited our ability to discern qualitative differences in ceratomyxosis among the Chinook stocks. While columnaris infection complicated our interpretation of Chinook mortality rate, over 70% of each Chinook group died prior to 19dpe with the majority of Chinook sampled after 11 dpe having clinical ceratomyxosis. Future studies directed at infection and disease progression will require larger exposure populations that allow for subsets to be monitored for mortality as well

as other subsets to be sampled for parasite detection over time. Additionally, better methods to control columnaris infections in juvenile salmonids of natural origin will need to be incorporated into future studies. In previous studies, we have shown that IGH Chinook are resistant to ceratomyxosis when exposed for a limited duration in the Klamath (Foott et al. 2007b). Both field collection and sentinel data indicates that actinospore concentrations in the Klamath River are often above a threshold level necessary to induce infection and disease in these locally-adapted stocks of Chinook salmon (Stocking et al. 2006, Nichols and True 2007).. The similar susceptibility of the Salmon R. and Shasta R. stocks to the hatchery stocks suggests that ceratomyxosis is a common limiting factor in Klamath R. basin salmon recovery.

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Appendix 1. Significant Comments to draft that were in addition to single word changes and minor editorial inserts.

Reviewer 1

1. 1st Paragraph in methods needs additional information as it currently lacks clarity. **Add reference to table 1**

2. Exposure section of methods - Confusing statement. Perpendicular or parallel to river flow?? Also, how are these cages anchored to the stream bed. Where within the stream bed were they placed? That is how far from the rivers edge were they placed and how deep was the water etc? **Agree – perpendicular to river edge**

3. Statistics methods - I presume your testing for proportions of these groups but not sure. Would be good to include a formal hypothesis. Please specify alpha level , (I assume 0.05)

Agree – language on significance level and rational for Fisher's test inserted

4. "While *C. shasta* trophozoites were observed in some of these early columnaris mortalities, it was difficult to evaluate the necrotic intestinal tissue and low parasite number limited our confidence in determining infection status of a given individual fish" - Explain more. This is not a good excuse **inserted language on rationale for limiting data set to 11-19dpe specimens**

Figure 3. Incidence of *P. minibicornis* infection (Infected) and infected with glomerulonephritis (clinical disease - Lacks clarity

Agree – change figure title language "infection (histo score =1)"

Reviewer 2

1. "histologically evaluated for their susceptibility to infection and disease progression by *Ceratomyxa shasta and Parvicapsula minibicornis.* All Chinook stocks had similar high levels of infection and disease by both parasites" - This may be clarified later, but is it accurate to say they are histologically evaluated for susceptibility to infection or for levels of disease? Would it be more accurate to use terms like **prevalence of infection and infection severity scores** which can be used as measures of disease? - **agree, changes made**

2. End of introduction - This might be a good place to describe how you use histology to characterize susceptibility. Especially important to tie in with and explain your infection vs disease ratings that you use below. We used a similar scale (but 1-5) in our Cowlitz study (attached manuscript in case you don't have) but didn't attempt to relate to the definitions as you have.

Agree – insert histo scoring language here

"At 19 days post exposure (dpe) all surviving fish were sampled" - Seems like a transition paragraph is missing – fish transported back to lab, held under same conditions? **Added language on subsequent holding period at lab.**

3. "statistical analysis was conducted using a Fisher Exact (2 Tail) Test. - what are you comparing? **Agree – language on significance level and rational for Fisher's test inserted**

3. "For all groups where there were sufficient survivors, we generated a mortality curve (Figs XX);= inserted comment - **this insertion was removed as only the coho and steelhead had large 19 dpe population numbers but had few mortalities.**

4. "Both hatchery and naturally produced Chinook progressed into a disease state over the 19 day holding period" - As measured by histol, mort, or combined? **The criteria for disease classification based on histological score was listed in the methods**

5. "Parasite invasion with tissue damage and inflammation (histology scores of 2 or 3) was seen in the majority of these fish." - .. Doesn't a histo score of 2-3 define disease? **Agree – remove sentence**

6. "Analysis between the Shasta River and Salmon River Chinook salmon revealed no difference in their high incidence of infection" - What were they? It would help to have a separate table showing % at each histo level – for both parasites.. It would give a little more data than the graphs.

Table 2 histological score summary inserted

7. "Iron Gate Coho appear to have similar susceptibility to *C. shasta* infection (86% incidence of infection) as Chinook (67 – 88%) but with slightly greater resistance to disease progression" - But disease progressed more slowly? Can you have resistance to disease progression?
Agree – remove "resistance" – state slower disease progression only