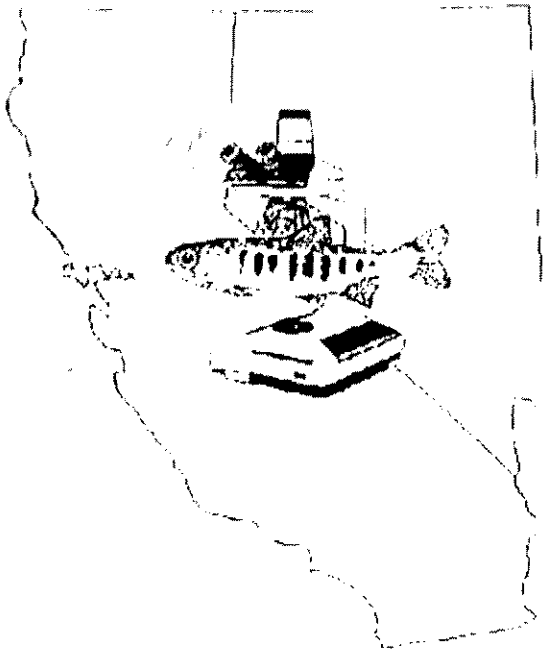
FY2002 Investigational Report:

Ceratomyxosis resistance in juvenile Chinook Salmon and Steelhead Trout from the Klamath River.



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Summary: Both mortality and clinical signs due to ceratomyxosis were similar between juvenile chinook salmon (*Oncorhynchus tshawytscha*) infected with *Ceratomyxa shasta* and reared at 16°C and 20°C. A 3 day (d) exposure in the upper Klamath River in June 2002 resulted in 100% infection in both temperature groups and ≥ 83% mortality within 17 d post-exposure. It is likely that the fish were overwhelmed by a highly infectious challenge. Non-specific defense mechanisms (phagocytosis, plasma complement and lysozyme activities) were not impaired in control fish reared at 20°C. Despite hypoproteinemia, plasma lysozyme activity was approximately 2X higher in infected salmon than control fish. Regardless of rearing temperature, salmon challenged with a single intraperitoneal injection of *Ceratomyxa shasta* trophozoites showed a strong inflammatory defense against the parasites. Juvenile steelhead (*Oncorhynchus mykiss*) exposed at the same site and reared at 14°, 18°, and 20°C did not develop ceratomyxosis. *Ceratomyxa shasta* was not detected in the steelhead by either PCR or histology. Salmon exposed along with the trout experienced typical ceratomyxosis. Both the salmon and steelhead were from Iron Gate hatchery and considered endemic Klamath R. stock. Rearing at temperatures up to 20°C does not appear to suppression non-specific immune functions but can accelerate ceratomyxosis in Klamath R. chinook salmon. Steelhead appear to be at low risk for ceratomyxosis when rearing or migrating in the Klamath River.

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Introduction:

Ceratomyxa shasta is a myxosporean parasite of salmonid fishes that occurs in a number of watersheds of the Pacific Northwest and whose lifecycle includes the polychaete, *Manayunkia speciosa*, as its intermediate host (Hoffmaster et al. 1988, Bartholomew et al. 1997). Infection can occur from spring through fall at water temperatures $\geq$ 7°C (Ching & Munday 1984, Hendrickson et al. 1989). Hendrickson et al. (1989) describe the parasite's distribution in California to include the San Joaquin, Sacramento, Pit, and Klamath River systems. High mortality due to ceratomyxosis has been observed in juvenile salmon out-migrants in the Klamath R. basin (Foott et al. 1999 and 2002). Elevated water temperatures in the Klamath system, commonly in excess of 20°C in the late spring and summer, act to accelerate the disease process (Udey et al. 1975). There is a large variation in susceptibility to ceratomyxosis among salmonid fishes with resistant strains being found primarily in enzootic drainages (Buchanan et al. 1983, Zinn et al. 1977, Iberra et al. 1991). A degree of resistance was documented in Klamath R. fall-run chinook salmon propagated at the Iron Gate Hatchery and exposed to infectious Pit River waters (Foott et al. 1999). This study examined the effects of elevated water temperature on resistance to ceratomyxosis and several non-specific immune functions of juvenile chinook salmon and steelhead challenged by exposure to infectious water.

Methods:

Fish handling - On June 21, 2002, four hundred juvenile fall-run chinook salmon (*Oncorhynchus tshawytscha*) from the California Department of Fish and Game Iron Gate Hatchery were placed in the Klamath River upstream of Beaver creek (262 rkm). Live box exposures in May 2002 at several upper Klamath R. locations had shown this site to be highly infectious. The salmon were held in river water (average 20.5°C) for 3 d within 0.03 m^3 live boxes and then transported to the California-Nevada Fish Health Center wet laboratory for extended rearing. These salmon are referred to as exposed (E) fish. Approximately 450 salmon (referred to as exposure controls {C}) were also held for 3 d within live boxes at the hatchery and transported to the wet laboratory at the same time. In order to reduce the occurrence of Columnaris disease, both groups received a 10 min., prophylactic bath of 1 ppm furanase upon arrival at the laboratory. The groups were dispersed as 24 fish replicates into 40L aquaria supplied with aeration and 8 L / min flow of either 16° or 20°C water. Each 1860 L system had twenty aquaria, a 760 L temperature conditioning tank with chillers and heaters, a 300 L effluent sump with chillers and heaters, and pumps. Freshwater inflow was set at 38 L / min. A commercial salmon diet was offered twice a day. Water temperature was monitored every 2 hrs with Onset™ Stowaway temperature loggers. Dissolved oxygen was measured with a YSI 95 meter and Hach kits were used for both pH and ammonia assays. The laboratory effluent was treated with 5 mg / L chlorine for 50 min, dechlorinated through activated charcoal filters, and discharged into a 1.3 ha abatement pond.

At 10d post-exposure (PE), intestinal content from 10 clinically ill fish were pooled and added to 5 mL cold Hanks Buffered Salt Solution with 1600 units / mL

Penicillin G – Streptomycin sulfate. A hemocytometer count of the suspension showed 285 trophozoites / μL as well as host cells and debris. Thirty uninfected control fish were given a 0.1 mL intraperitoneal injection of the suspension and divided between 16° and 20 °C aquaria (fish identified as IPE). Injection controls (IPC) received 0.1 mL of HBSS only.

On July 19, 2002, two hundred juvenile steelhead (*Oncorhynchus mykiss*) were exposed for 3 d to river water (average 23.2 °C) at the same Klamath R. site. One live box of 30 chinook salmon was also exposed at the site and the salmon later reared at 20°C. Only 59 steelhead were recovered from the live boxes for the experiment. We assume the other steelhead escaped from a tear in the netting of 2 of 3 live boxes. Exposure control steelhead (C) were held at the hatchery and transported to the wet laboratory at the same time as the exposed group. These fish were handled in a similar manner as the salmon in the June experiment except only 4 - 8 fish per group were placed into replicate aquaria maintained at 14°, 18°, and 20°C. No injection challenges were performed on the steelhead. Both the chinook salmon and steelhead are progeny of Klamath R. stock adults that returned to the hatchery.

Necropsy – Fish were euthanized by an overdose of MS222, measured for fork length and weight, and examined for pale gill (anemia) and any external abnormality such as swollen abdomen or hemorrhagic vent. Fulton condition factor was calculated from the fork length (KFL = weight / (fork length) ^{3} x 10^5). The caudal peduncle was then cut and blood collected into heprinized microhematocrit tubes. The tubes were centrifuged at 10,000 x g for 5 minutes, a hematocrit measurement taken, and the plasma stored frozen on dry ice in two aliquouts (15 μL complement assay, and 12 – 20 μL for lysozyme and total protein). Protein determination was the only assay conducted on steelhead plasma due to volume limitation. Plasma samples were stored at -70°C until assayed. In the control salmon, gill tissue was collected for ATPase assays. Upon dissection, internal abnormalities such as intestinal hemorrhage, or swollen kidney were noted. The intestinal tract and kidney was fixed for histological examination. Prior to fixation, a 1- 2 mm section of lower intestine was removed by DNA-free tools (bleached and 2X washed scalpels and disposable sticks) for storage at -70°C as a PCR archive sample. Several intestinal samples from exposed steelhead, that did not show clinical or histological signs of *C.shasta* infection, were tested for specific parasite DNA by the Polymerase Chain Reaction (PCR) method of Bartholomew (2001).

Histology – Intestinal tract (including pyloric caeca) and kidney was rapidly removed from the fish after blood sampling and placed in Davidson's fixative, processed for 5 μm paraffin sections and stained with hematoxylin and eosin (Humason 1979). All tissues for a given 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) without knowledge of the sample group.

Plasma lysozyme and total protein – The lysozyme activity of plasma (mOD / min.) was determined from 5 μL samples frozen on dry ice, stored at -70°C, and later assayed by the turbidimetric method at pH 6.4 (Ellis 1990). Despite its lower activity at pH 6.4, a hen egg white lysozyme (Sigma Chemical Co.) standard curve was run on each plate (Findlay and Munday 2000). Total protein concentration (g / dL) of the same plasma sample was assayed by the biuret method (Sigma Chemical Co. kit No. 541).

Plasma complement activity - Hemolytic activity, of the alternative complement system, against rabbit erythrocytes was assayed by the method of Alcorn et al. (2002). Briefly, a 15μL aliquot of plasma was diluted 15X in buffer (0.1% gelatin, 0.1 M EGTA, 0.1 M $MgCl_2$ in veronal-buffered saline), and reacted with 1% Rabbit red blood cells for 60 min at 15°C. The hemoglobin content of the reaction well was determined by the absorbance at 540 nm. Percent hemolysis was calculated as follows:
{(Mean OD sample – Mean OD sample background) – (mean OD neg. control – mean OD Neg. control background) / (Mean OD 100% hemolysis control – mean OD hemolysis background)] * 100

Kidney phagocyte bactericidal assay - The method of Secombes (1990) was used with a single cell suspension of anterior kidney cells and *Yersinia ruckeri* . Briefly, the anterior kidney was aseptically removed from fish that had been bled and placed into ice cold Eagles Minimum Essential Medium (MEM) with 5% fetal bovine serum and 100 unit /mL penicillin G and streptomycin sulfate (MEM5). Approximately 24 hrs later, the refrigerated kidney was disrupted by repeated titration with a 3CC syringe and 21 G needle. The suspension was washed by centrifugation, resuspended in 1 mL MEM5, a hemocytometer count performed on a sub-sample in 0.2% trypan blue solution, and the suspension adjusted to 2×10^6 leukocytes / mL. Dead cells and erythrocytes were excluded from the counts. Samples with greater than 30 % dead cells were excluded from the assay. A 96 well plate was loaded with 100 μL kidney cells and 20μL of a 24 hr old 10^7 *Y.ruckeri* / mL suspension. After 3 hr incubation at 15°C, the kidney cells were lysed in 0.2% tween 20 and the surviving bacteria allowed to grow in Tryptic Soy Broth (TSB) for 18 hrs. The bacterial number per well was then estimated by an Optical density reading at 600 nm following the addition of the mitochondrial dye MTT (3(4,5-dimeththiazoly-2-yl 2,5 diphenyltetrazolium bromide). Percent kill was calculated as: (mean OD of triplicate sample wells – OD TSB-MTT blank) / mean OD bacteria only wells – OD TSB/MTT blank).

ATPase - Gill Adenosine Triphosphatase activity (ATPase = μmoles ADP / mg protein / hr) was assayed by the method of McCormick and Bern (1989). Briefly, gill lamellae were dissected and frozen in sucrose-EDTA-Imidazole (SEI) buffer on dry ice. The sample was later homogenized, centrifuged and the pellet sonicated prior to the assay. ATPase activity was determined by the decrease over time in optical density (340 nm) as NADH is converted to NAD+. This activity was reported as μmole ADP / mg protein / hr as 1 mole of NAD is produced for each mole of ADP generated in the reaction.

Results:

Water quality -Rearing temperatures for both salmon and steelhead were maintained within 0.5°C of their respective target temperature. Other water quality parameters were maintained within normal ranges for salmonids: dissolved oxygen ranged from 5.7 – 9.6 mg /L, pH from 7.5 – 8.0, and unionized ammonia was below 0.05 mg / L.

Chinook - Rearing temperature did not alter the mortality pattern among the exposed groups. The first mortality, associated with ceratomyxosis, occurred 7 d post-exposure (PE) with the peak mortality occurring at 13 and 14 d PE for the 20°C and 16°C exposed fish, respectively (Fig. 1). The experiment was concluded at 17d PE as there were only 8 live fish remaining in each exposed group. Cumulative percent mortality for the 16°C exposed group was 83% (155 dead of 188 total) and 90% (212 dead of 236 total) for the 20°C group. No mortality occurred in the control groups. Ten fish among the exposed groups were observed at 7d PE to have clinical signs of Columnaris (*Flavobacterium columnare*) and were removed from the experiment. Both control and exposed groups were then treated with a second 30 min static bath of 1% furanase. No additional observations of Columnaris were made for the remainder of the experiment. An *Ichthyophthirius multifiliis* (Ich) infestation was detected in fish held at both water temperatures on the final 17d PE sample. In particular, both exposed and control salmon in the 20°C system had the characteristic white spot and displayed "flashing" behavior.

Mean fork lengths ranged between 95 - 103 mm with mean weights ranging between 9.0 – 11.3 g (Table 1). Anemia, as indicated by hematocrits below 20%, was observed in 50% of the exposed fish in both temperature groups at 17 d PE. Mean hematocrit values for the controls ranged from 33 – 36% (Table 1). Multifocal hemorrhages were consistently observed within the intestinal tract of all exposed fish (Table1). In histological sections of the intestinal tract, *C. shasta* trophozoites were seen primarily in the small intestine and pyloric ceca of all exposed fish (Fig 2.). The infection was associated with severe hyperplasia of mononuclear cells within the lamina propria, hemorrhage, and erosion of the intestinal epithelium (Fig. 3a and 3b). The intestinal lumen was often filled with necrotic host cells and trophozoites. By 17d PE, trophozoites had often penetrated the intestinal tract and the infection had spread systemically in the fish. At this point, trophozoites were observed within inflammatory foci in the kidney, liver, and peripancreatic region.

Figure 1.
Ceratomyxosis mortality curve for juvenile chinook salmon exposed for 3 d to the Klamath R. and reared at 16° and 20°C. Cumulative percent mortality for fish held at 16° and 20°C was 83% and 90%, respectively.

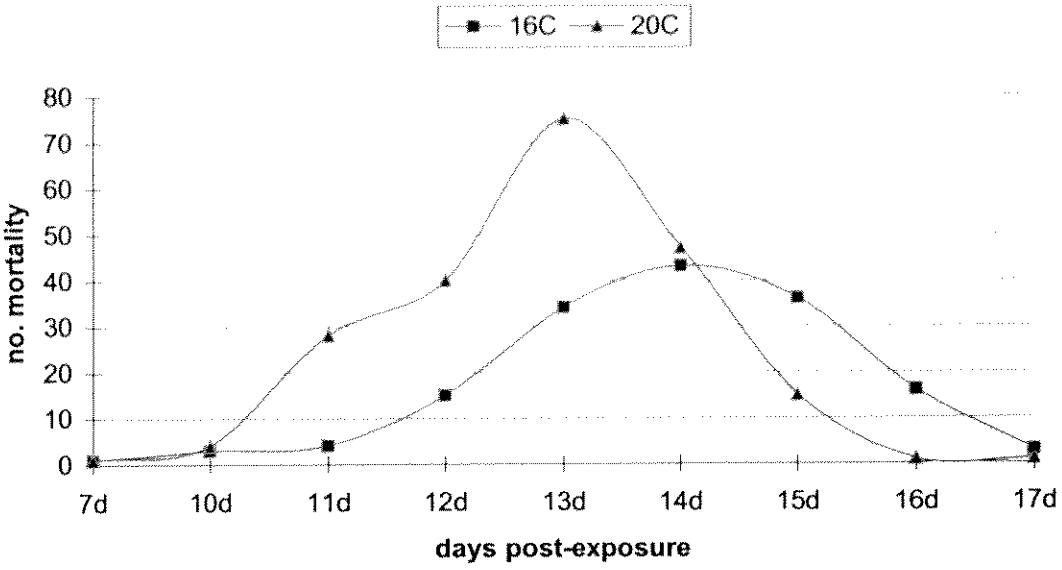


Figure 2. *Ceratomyxa shasta* trophozoites (bar = 20μm).

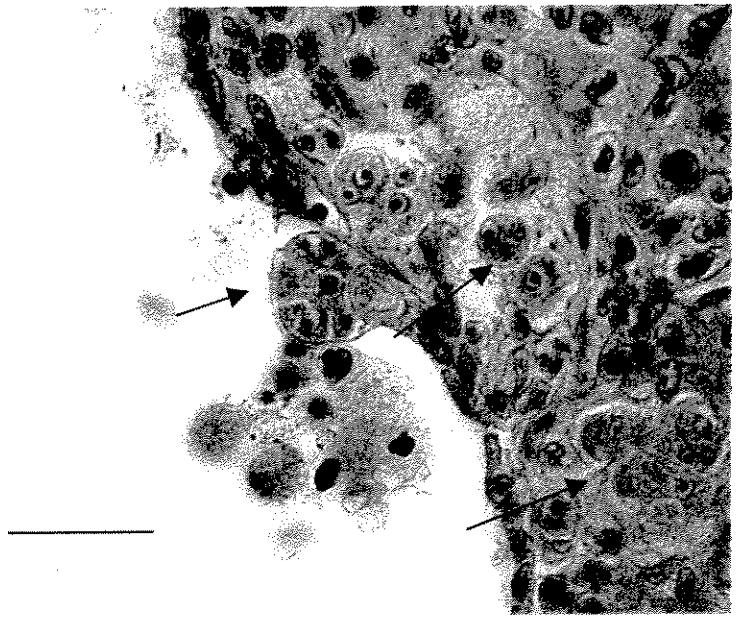


Table 1. Fork length (FL), weight (WT), hematocrit (HCT), and prevalence of clinical signs in exposed (E) and control (C) salmon sampled at 10 and 17d post-exposure. Salmon given a 0.1 mL intraperitoneal injection of either *C.shasta*-infected intestinal homogenate (IPE) or Hanks Balanced Salt Solution (IPC) were sampled 7d post-injection. Non-prevalence data reported as mean ± standard error.

Group	FL (mm)	WT (g)	HCT (%)	Pale gill	Hemorrhagic Intestine	Swollen Kidney
E16-10d	102 ± 2	10.6 ± 0.6	44± 2	10%	100%	20%
E20-10d	98 ± 2	9.6 ± 0.5	35 ± 2	0%	100%	20%
C16-10d	98 ± 2	10.3 ± 0.5	36 ± 3	0%	0%	0%
C20-10d	99 ± 2	10.8 ± 0.5	33 ± 1	0%	0%	0%
E16-17d	99 ± 2	9.2 ± 0.6	19 ± 2	38%	100%	40%
E20-17d	102 ± 3	10.3 ± 0.8	20 ± 3	0%	100%	10%
C16-17d	101 ± 1	11.3 ± 0.4	34 ± 1	0%	0%	0%
C20-17d	97 ± 2	10.2 ± 0.6	35 ± 1	0%	0%	0%
IPE16	95 ± 3	9.0 ± 0.7	31 ± 4	0%	20%	0%
IPE20	98 ± 2	10.2 ± 2.4	31 ± 4	0%	30%	0%
IPC16	103 ± 2	10.6 ± 0.6	36 ± 1	0%	0%	0%
IPC20	101 ± 2	10.8 ± 0.6	31 ± 2	0%	0%	0%

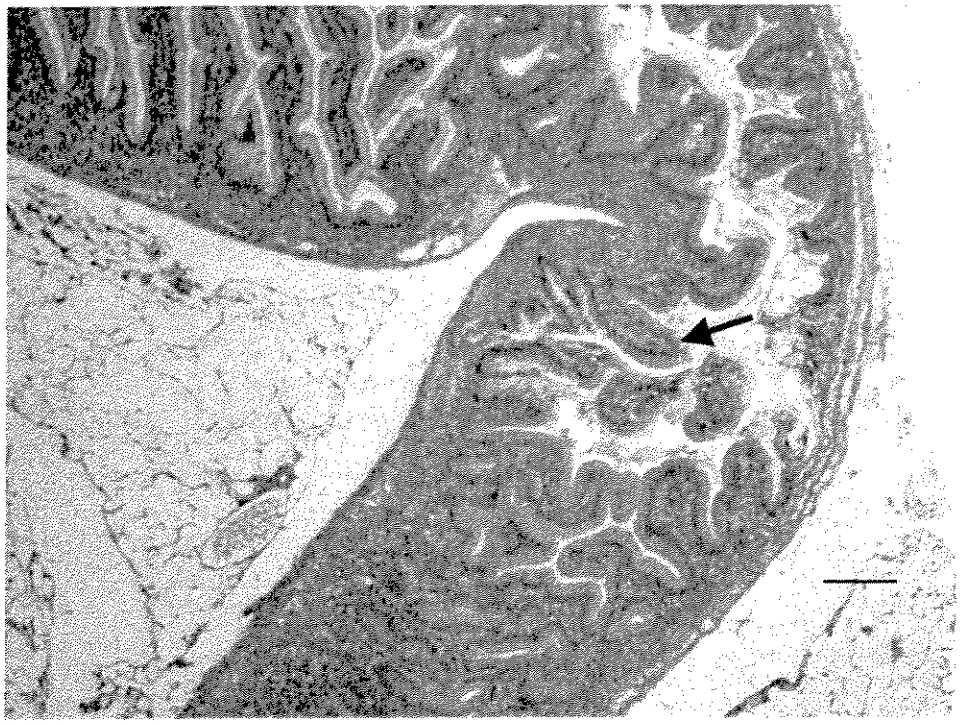


Figure 3a. Section of normal intestine with intact epithelium (arrow). H & E stain, bar = 100 μm.

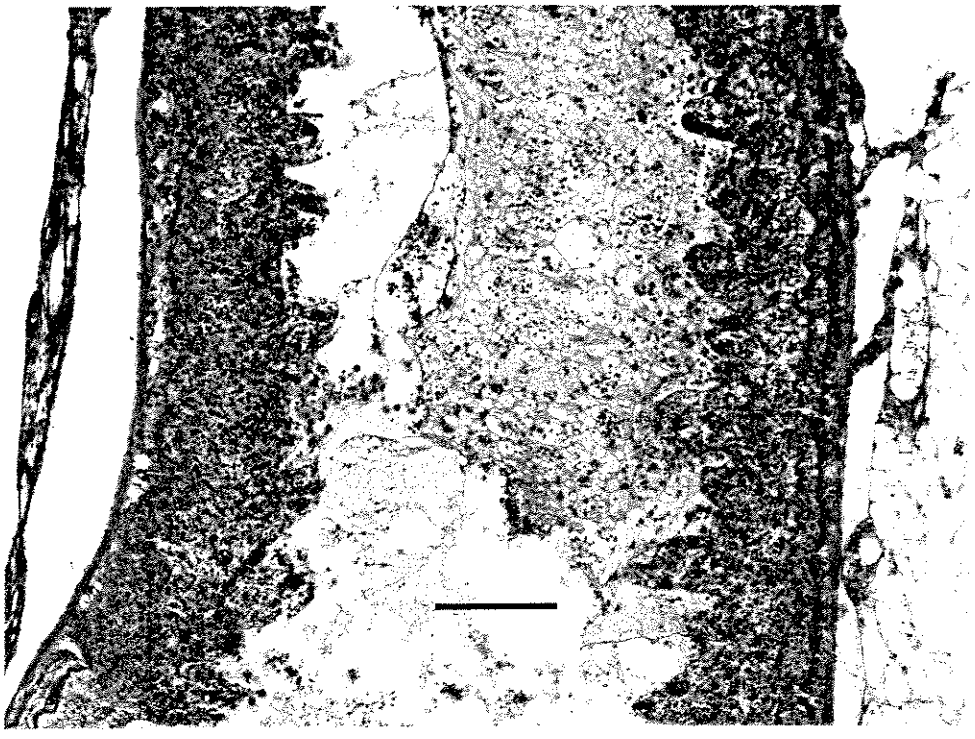


Figure 3b. Severe ceratomyxosis. Note loss of epithelium layer and cellular debris within lumen. (H&E stain, bar = 100 μm).

All injected fish were sampled at 7d post-injection (PI). No mortality occurred in either parasite or HBSS injected salmon during the 7 d period. No parasites were observed in kidney and intestinal tract sections from the HBSS – injected control fish. In contrast to the river exposure fish, relatively few *C.shasta* trophozoites were observed in tissue sections from 4 of 9 IPE-16°C (44%) and 5 of 10 IPE-20°C (50%) salmon. These parasites were associated with an intense inflammatory response on the serosal surface of the intestinal tract, and in blood sinuses of the kidney and liver. No *C.shasta* trophozoites were observed within the intestinal tract of the injected fish. It appears that the limited challenge titer was successfully resisted in several fish, as the few parasites observed in their inflammatory cell foci were necrotic. Two of 10 IPE-20°C fish appeared to have advanced ceratomyxosis as indicated by high trophozoite numbers and peritonitis.

Another myxosporean parasite was seen in the kidney sections of all exposed salmon (Fig. 4a and 4b). It is commonly observed in kidney sections from salmon collected throughout the Klamath basin (Foott et al. 1999 & 2002). The myxosporean differed from *C.shasta* in staining characteristics of the cytoplasm and nuclei. In hematoxylin and eosin stained sections, *C.shasta* trophozoites have basophilic cytoplasm and nuclei that contain a large eosinophilic karyosome with distinct peripheral chromatin (Fig. 2). The kidney myxosporean was most prevalent in the glomeruli and often associated with glomerulonephritis in the 17d PE samples. Gross swelling of the kidney was observed in 33% of the infected 17d PE fish. No parasites were observed in the kidney or intestinal tracts of exposure control fish.

Plasma protein concentrations were significantly (ANOVA, $P< 0.01$) reduced in exposed fish and by 17d PE were less than 1.0 g /d L (Fig. 5). There was no significant difference in the plasma protein levels between *C.shasta* injected or HBSS-IP controls sampled at 7d PI. The mean protein values for all injected groups were above 2.1 g/dL. Despite the low plasma protein levels of infected exposure salmon, both plasma lysozyme and complement activities tended to be elevated. Lysozyme activity (mOD / min) was approximately 2X greater in the exposed fish compared to the controls (Fig. 6). Elevated lysozyme activity was seen in the 20°C control group sampled at 17d PE (Fig. 6). Salmon challenged by injection of the parasite also had elevated lysozyme activity values regardless of rearing temperature (Fig. 7). Similar to the 20°C exposure control fish at 17d PE, elevated lysozyme activity was seen in HBSS- injection controls held at 20°C (Fig 7). The HEWL equivalent units (pH 6.4) ranged from 3.0 – 59.6 µg / mL in the data set. Despite a 1.6 to 1.7X lower plasma protein concentration, complement hemolytic activity (% hemolysis) was similar among both (E) and (C) fish from both temperatures at 10d PE (Fig. 8). The mean % hemolysis declined over time in all groups and the exposed fish were significantly lower than their control cohort group at a similar temperature. The severe hypoproteinemia of the 17d PE infected exposure groups was a probable factor in the low hemolytic activities of these fish. There was no clear correlation between plasma protein concentration and % hemolysis (r^2 =0.07 and 0.21 for 10d and 17d groups, respectively).

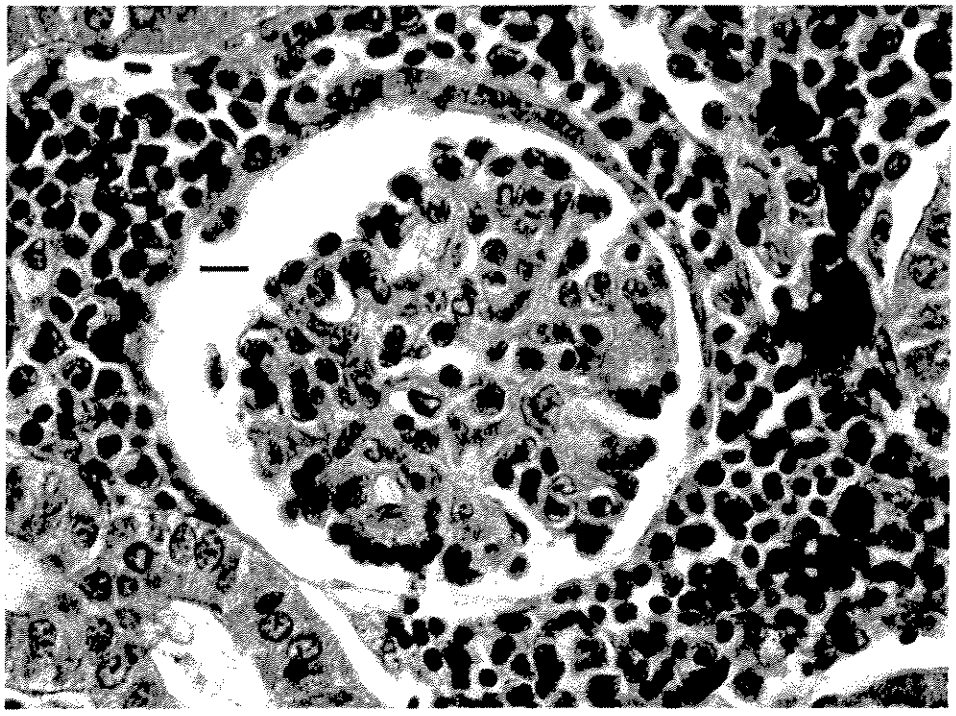


Figure 4a. Normal glomerulus in salmon kidney (bar = 5 μm).

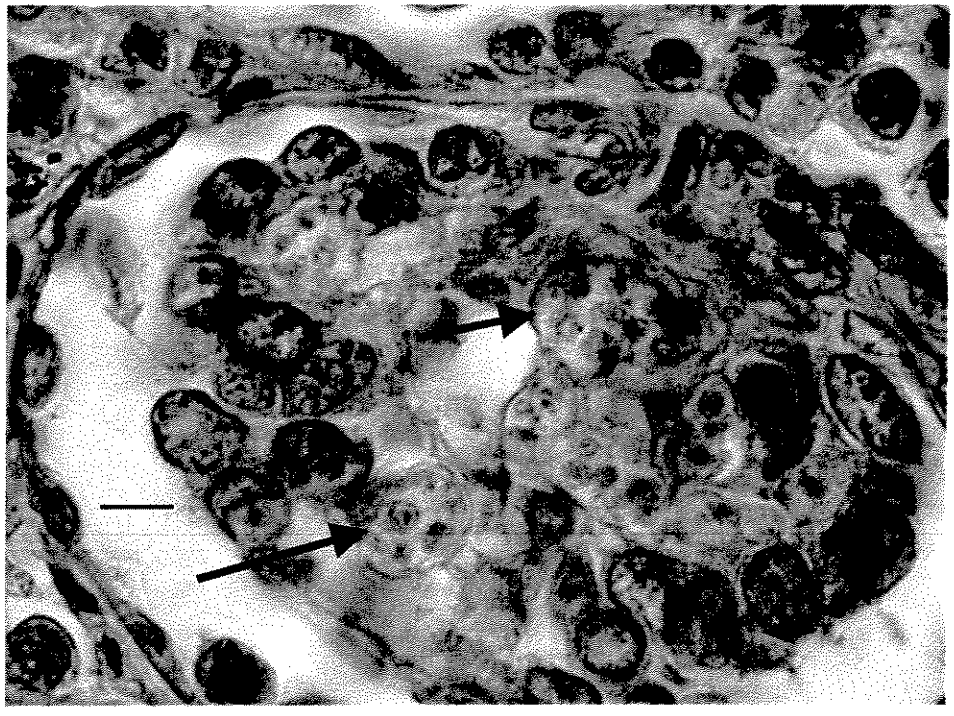


Figure 4b . Kidney myxosporean (arrows, bar = 5μm) in kidney glomerulus.

Figure 5. Mean plasma protein concentration (g/dL) of exposed (e) and control (c) salmon held at 16° and 20°C. Fish were sampled at 10 and 17d post-exposure.

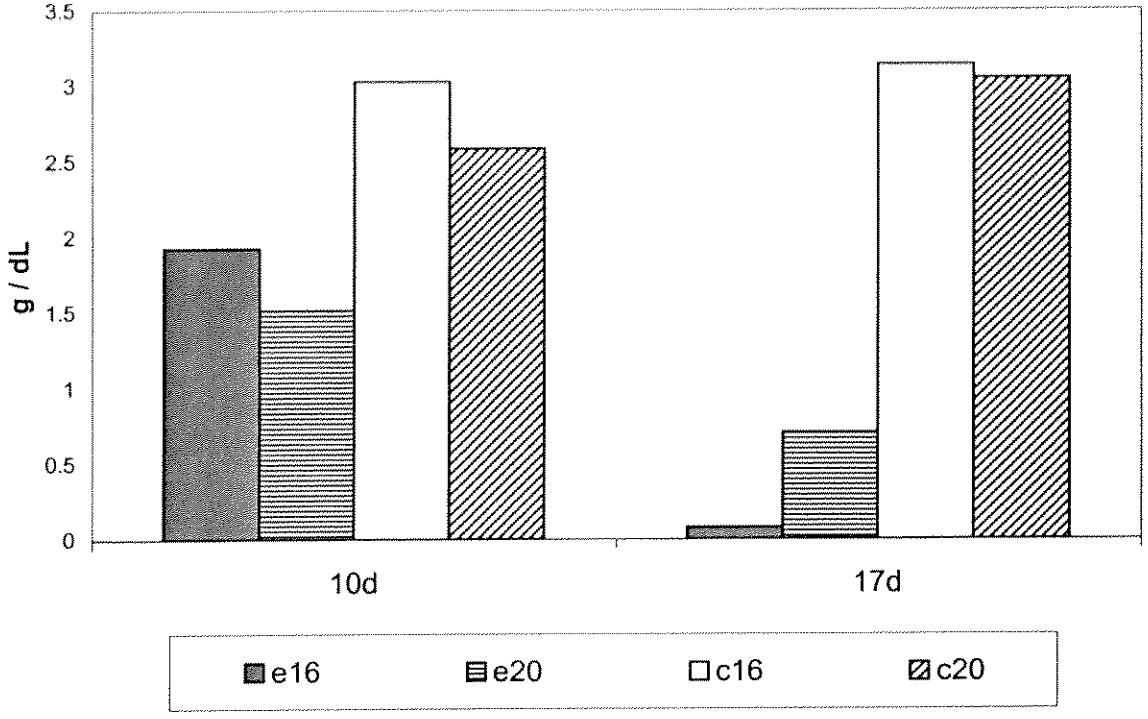


Figure 6. Mean plasma lysozyme activity (mOD / min) of exposed (e) and control (c) salmon held at 16° and 20°C. Fish were sampled at 10 and 17d post-exposure.

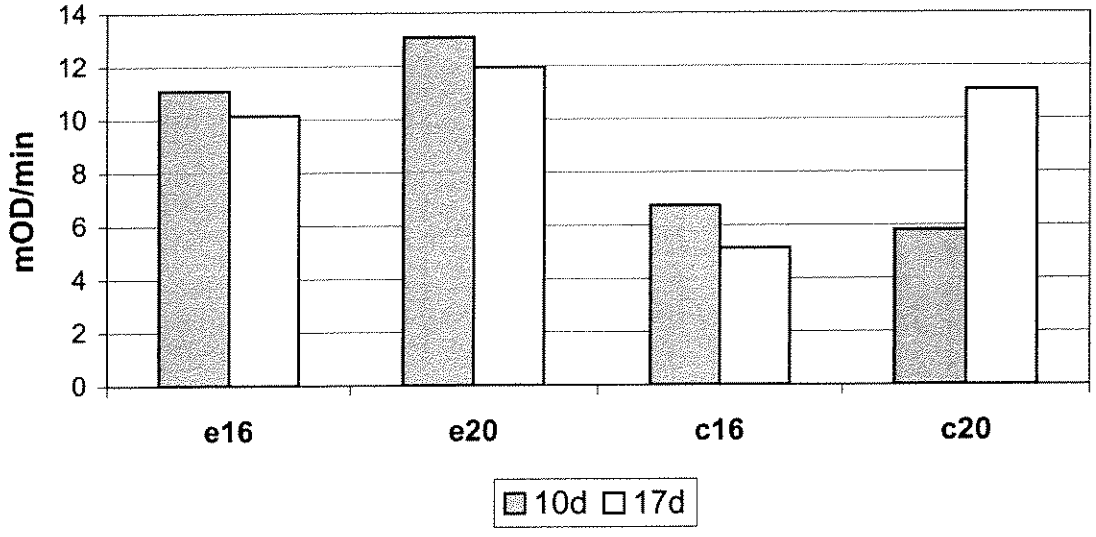


Figure 7. Mean plasma lysozyme activity (mOD / min) of parasite injected (IPE) and HBSS - injected control (IPC) salmon sampled 7d post-injection.

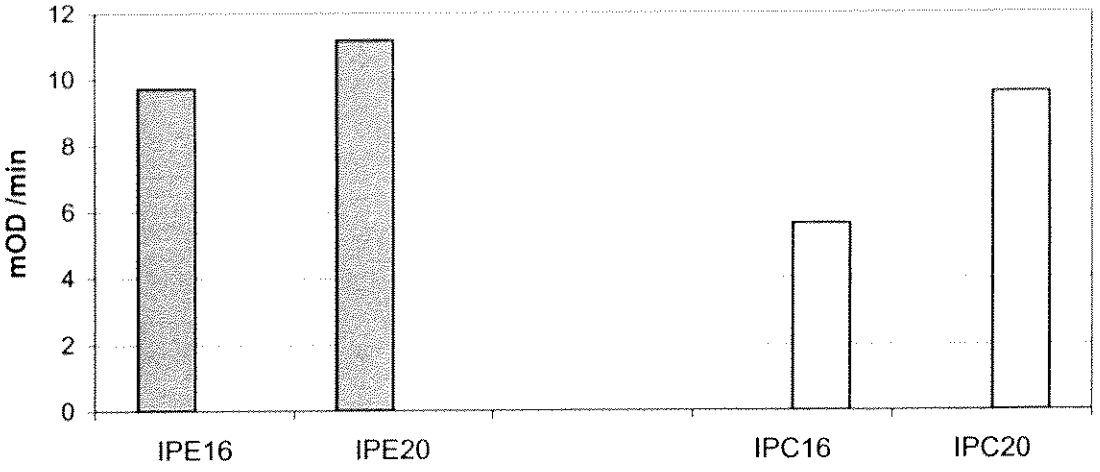
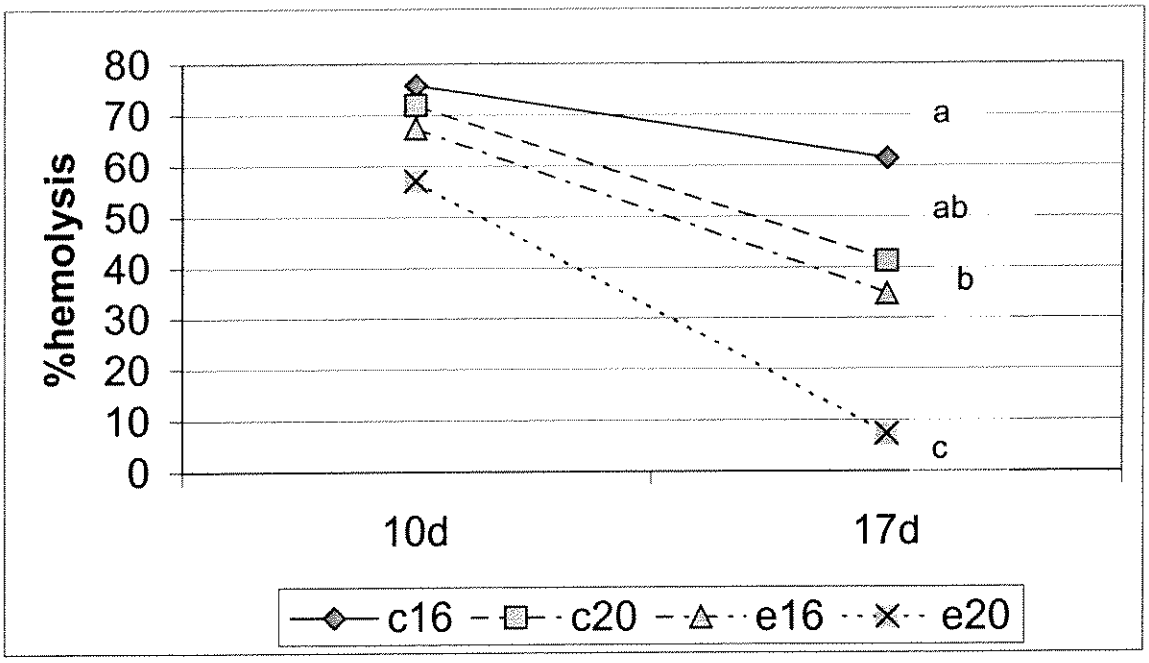


Figure 8. Hemolytic activity of 15 μL plasma samples from control (C) and exposed (E) salmon held at 16° and 20°C. Alternative complement action against rabbit erythrocytes recorded as mean percent hemolysis. Letters indicate statistical difference (ANOVA, P<0.01).



Hemolytic activity of parasite injected salmon were not significantly different than the controls (Fig. 9).

Phagocytic activity of anterior kidney cells, as measured by the MTT bactericidal kill ratio, was similar between exposed and control salmon at the same rearing temperature. The 20° C groups had higher % kill values than the 16 °C fish. At 10d PE, the mean % kill (± SEM) of the 16° C exposed and control fish were 66 % ± 6 and 72 % ± 4, respectively. In comparison, the 20° C exposed fish had a mean % kill of 98% ± 0.2 and the controls 99 % ± 0.2. At 17d PE all groups had ≥ 98 % kill values. The ratio of *Y. ruckeri* bacteria to phagocyte appeared to be too low for the 17d assay.

Gill ATPase activity was reduced in control salmon held at 20°C compared to their 16°C cohorts (Fig. 10). By 17 d PE, this difference was statistically significant (ANOVA, $P < 0.05$).

Steelhead - No mortality or clinical signs of infection occurred in either exposed or control steelhead groups over the 24 d experiment. Mean fork length tended to increase over the experiment in all groups (Fig. 11). Average group weight ranged from 1.5 – 3.1 g with condition factors ranging from 0.934 – 1.220. Hematocrits were above 30% and plasma protein concentration tended to increase over time in all groups. There were no significant differences in plasma protein between temperature groups at any given sample date (Fig. 12). No *C. shasta* trophozoites or myxosporean kidney parasites were observed in histological sections from 47 exposed and 21 control steelhead sampled throughout the experiment. Samples of lower intestine from 3 steelhead collected immediately after the 3 d exposure and two steelhead sampled at 10d PE were negative by PCR assay.

Both of the above parasites were seen in histological sections from chinook salmon exposed along with the steelhead and sampled at 6 and 13d PE. Unlike salmon exposed in June and sampled after10d PE, only limited numbers of trophozites were observed in the epithelium of the small and large intestine from 3 of 6 salmon exposed in July and sampled at 6d PE. These early infections were associated with localized inflammation but no necrotic changes were observed in the intestine. A lower intestine sample from a salmon collected at 6d PE was negative by PCR despite the presence of several trophozoites in histological sections of the pyloric ceca. All 3 salmon sampled at 13 d PE, had severe ceratomyxosis and two were also infected with the kidney myxosporean.

Figure 9. Hemolytic activity of 15 μL plasma samples from injection controls (ipc) and *C.shasta* injected (ipe) salmon held at 16° and 20°C. Alternative complement action against rabbit erythrocytes recorded as mean percent hemolysis.

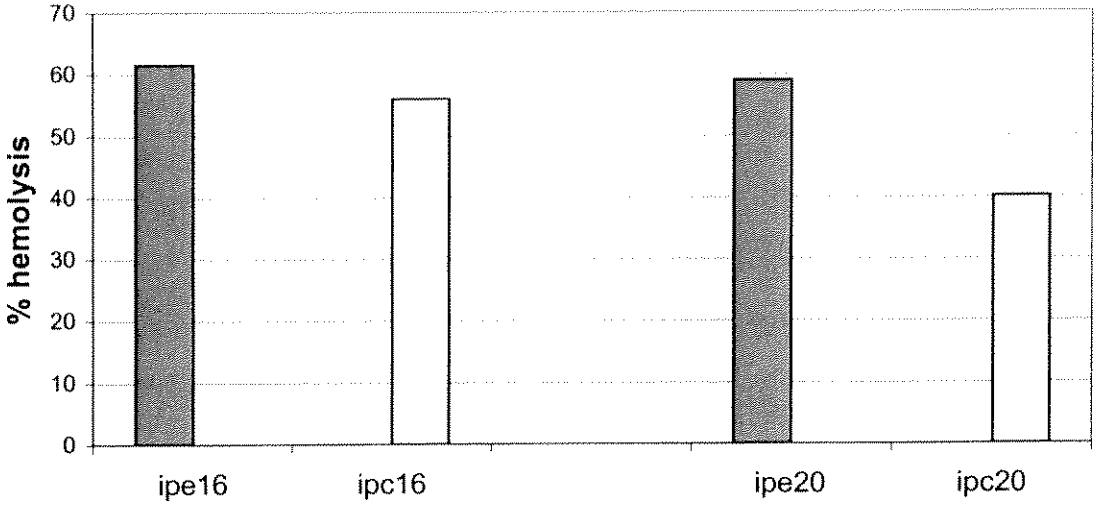


Figure 10. Mean gill ATPase activity of control salmon (c) held at 16° and 20°C, and sampled at 10d and 17d into the experiment. Letters indicate significant differences (t-test, P< 0.01).

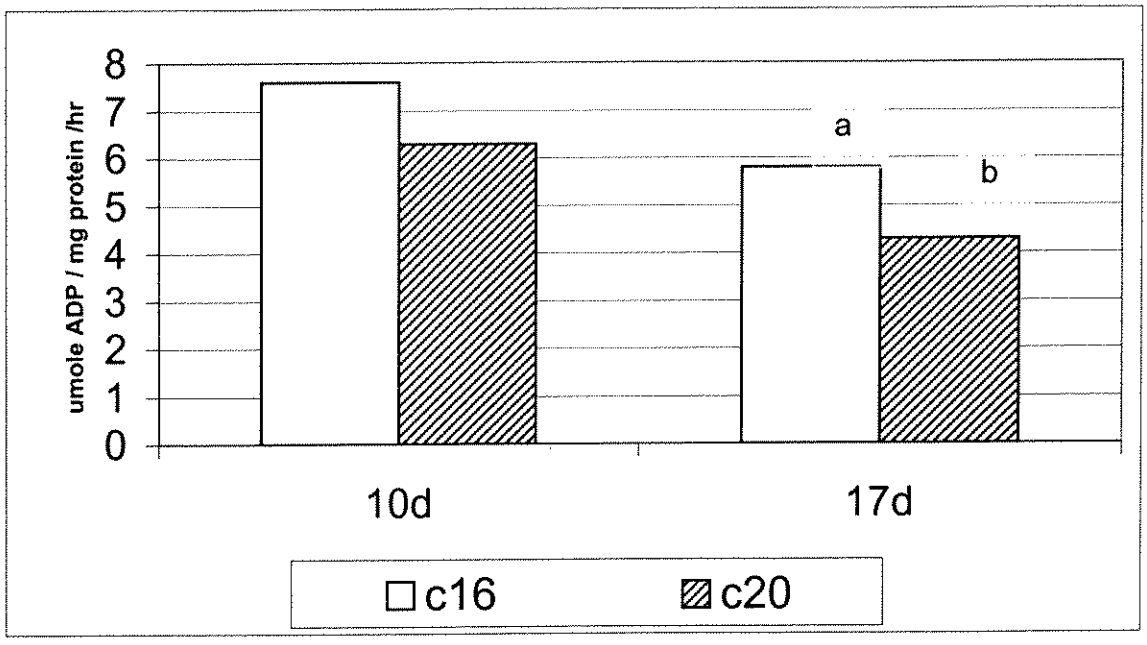


Figure 11. Mean fork length of exposed (e) and control (c) steelhead sampled at 3 (upon return from exposure sites),10, 17 and 24 d post-exposure. There was no sample from 14°C control fish on day10 PE.

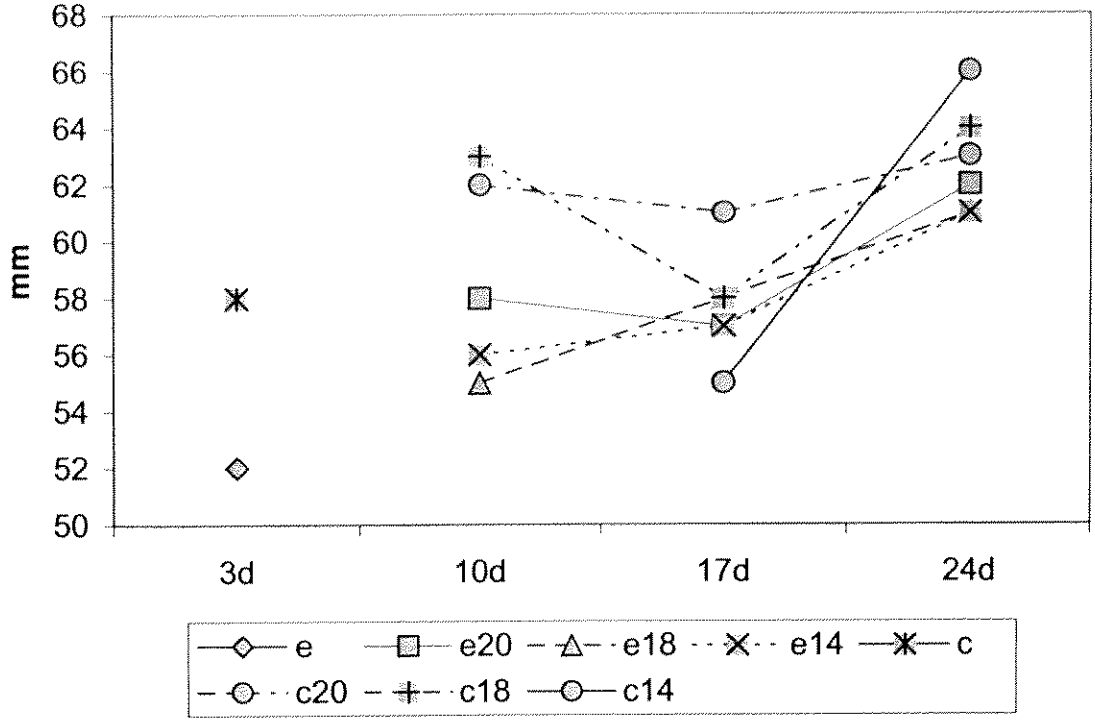
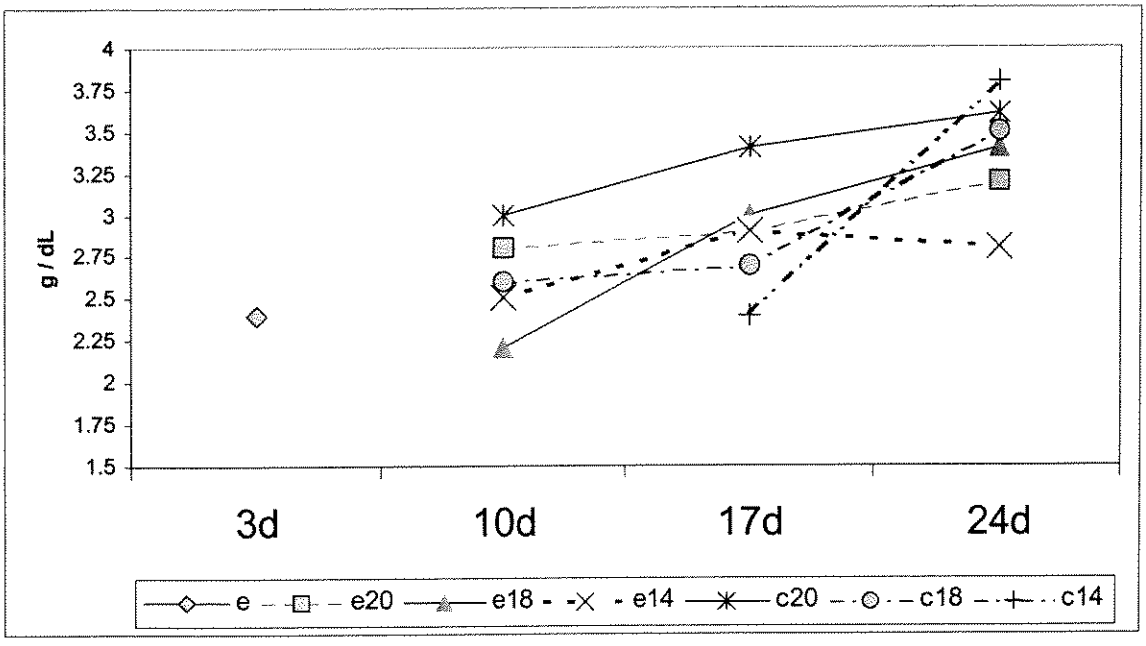


Figure 12. Mean plasma protein concentration (g /dL) of exposed (e) and control (c) steelhead reared at 14°,18°, and 20°C. Fish sampled at 3,10,17, and 24 d post-exposure. There was no sample from 14°C control fish on day10 PE.



Discussion:

There are numerous reports of resistance to ceratomyxosis by endemic stocks of salmonids (Buchanan et al. 1983, Ibarra et al. 1991, Zinn et al. 1977). Juvenile chinook salmon and steelhead from Iron Gate Hatchery demonstrated a similar pattern of resistance to *Ceratomyxa shasta* in 1995 and 1996 exposure study at the California Department of Fish & Game Crystal Lake hatchery. When groups were exposed for 20 - 85 d to *C.shasta* infectious water from the Pit River, none developed ceratomyxosis (Foott et al. 1999). Water temperature during the 1995 and 1996 exposures were below 17°C and the challenge titer of the water was unknown. No mortality or histological signs of infection were detected in the Iron Gate fish. Control rainbow trout (Shasta strain), exposed along with the Iron Gate fish, suffered complete mortality due to ceratomyxosis. The results of this limited study are not consistent with observations of juvenile chinook salmon captured in the Klamath River and estuary. Additionally, Bartholomew et al. (2001) report that a strain of rainbow trout obtained from the Oregon Department of Fish and Wildlife and identified as "Klamath strain" was highly resistant to exposure challenge by *C.shasta* as well as the F1 hybrid cross with a susceptible trout strain.

The high incidence of ceratomyxosis in chinook smolts collected from the Klamath River and estuary indicate that this parasitic disease is a major mortality factor for both hatchery and natural populations. Over 40% of chinook smolt samples collected from the Big Bar rotary screw trap (rkm 81) have been diagnosed with severe ceratomyxosis (Foott et al. 1999 & 2002). The Klamath River would appear to have all the elements necessary for the parasite's lifecycle: specific habitat(s) for the intermediate polychaete host (*Manayunkia speciosa*) a temperature profile that facilitates infection and accelerates the disease process, and the continual re-infection of the polychaetes from spores shed by infected salmonids (particularly adults).

While Klamath R. salmonids face the greatest threat, there is evidence Trinity R. salmon smolts become infected and diseased after entering the Klamath on their out-migration. In the summer of 2002, twenty-two percent of marked Trinity R. hatchery chinook salmon smolts collected in the estuary were infected with *C.shasta* (unpublished data, report in progress, CA-NV Fish Health Center). It is unlikely that many smolts with detectable infection survive the disease. Ceratomyxosis can persist in juvenile salmon after their saltwater entry (Margolis & Evelyn 1975).

In our study, the magnitude of parasite challenge appeared to influence disease severity more than rearing temperature. While the mortality rate of 20°C fish was slightly greater than the 16°C group, there was no appreciable difference in the level of severe ceratomyxosis experienced by both groups. The ecological outcome (death) for salmon challenged at such levels would be similar under moderate spring temperatures as well as higher summer temperatures. Neither group demonstrated resistance to infection. Initial infections were consistently observed in the small intestine and pyloric ceca while lower intestine involvement tended to occur at 13d PE. It may be necessary to sample both small and large

intestine to optimize PCR detection of early infections. The high incidence of *C. shasta* infection and rapid progression into clinical disease suggests the salmon were subjected to a super-infection as a result of exposure to high concentrations of infectious actinospores in the river. Salmon injected with limited numbers of the parasite may have been more successful in controlling infection. It is not known how many of the injected trophozoites were viable and infectious. While two injected 20°C fish were diseased, eight others in this group had either no detectable infection (by histology) or appeared to be controlling the infection. Fish in the latter category had degenerative parasites within inflammatory foci. Unfortunately, the Ich outbreak necessitated ending this aspect of the study at 7d PI.

Dose-related liabilities to *C. shasta* in resistant stocks have been documented in the literature. Ibarra et al. (1992a) reported that progeny from crosses of resistant and susceptible trout parents showed intermediate levels of resistance to ceratomyxosis when challenged by a 7 d exposure to infectious waters. These same F1 hybrids experienced significantly higher mortality due to ceratomyxosis when challenged by continuous exposure (>120 d). Similarly, intraperitoneal injection of large numbers of trophozoites and pre-sporogonic stages into resistant strains of trout resulted in lethal ceratomyxosis and was dose-dependent (Ibarra et al. 1991). Such a challenge would bypass the epithelial defenses of the intestinal tract.

Despite being held at 20°C, chinook salmon exposed along with the steelhead in late July were slower to develop clinical ceratomyxosis than salmon exposed in June. Water temperature during the June exposure was 20.5°C while the July exposures ranged from 21° – 23°C. It is possible that the concentration of infectious actinospores was lower in July than June due to elevated temperature effects on the parasite or its polychaete host. Markiw (1992) reported the actinospores of *Myxobolus cerebralis* became non-viable within 3 d at 19° - 20°C compare to 7d at 7°C. Ratliff (1983) reported that *C.shasta* infections occurred in sentinel trout held below the thermocline in Lake Simtustus (Oregon) but not in cohorts held in the warm surface waters (≥ 18°C). The author postulates that the infective stage was associated with the bottom and could not penetrate the thermocline. Another explanation is that high water temperatures limit the infectivity of the actinospore. If high summer temperatures limit infectivity, seasonality of infection may be somewhat bi-modal in the Klamath system with peaks in the early spring and fall.

Suppression of non-specific immune defenses did not appear to be responsible for the poor resistance observed in the salmon. Rapid control of any parasites that succeed in invading the intestinal tract would likely be a combination of non-specific cellular and humoral mechanisms. The role of activated macrophages in protection from parasitic infection has been described for other fishes (Whyte et al. 1990, Jones & Woo 1987, Buchmann & Bresciani 1999). The exact mechanism(s) for ceratomyxosis resistance has not been described in the literature. Bartholomew et al. (1989) reported that they could not detect a

specific immune response (immunoglobulin) in salmonids naturally infected with *C.shasta*. The authors speculate that exclusion of the parasite from the intestine is the primary defense mechanism but that super-infection can overwhelm this defense. The non-specific defense mechanisms measured in control salmon were not impaired by rearing at 20°C. Additionally, the non-specific defenses of infected fish were either elevated or at levels similar to the controls until they were near death. When sampled at 10d PE, the infected salmon demonstrated elevated lysozyme activities and similar phagocyte killing ability and complement activities as the controls. While complement activity did decline at 17 d PE, the infected fish had extremely low plasma protein concentrations. Despite their moribund condition, phagocyte killing ability and lysozyme activity remained high in these 17d PE infected salmon. The elevated lysozyme activities observed at 17d PE in control salmon held at 20°C was a likely response to their Ich infestation. We have observed similar high lysozyme activity values in juvenile chinook salmon from the Merced R. hatchery infected with the kidney parasite *Tetracapsula byrosalmonae* (formerly PKX). Lysozyme is produced by both macrophages and neutrophils in fish, and increased serum activity has been associated with activation of these phagocytes by infection (Paulsen et al. 2001, Munoz et al. 2000, Balfry et al. 1997).

Salmon exposed to Klamath River water were also infected with a second myxosporean species that induced glomerulonephritis. Dual infection is a common condition for juvenile chinook salmon migrants in the Klamath R. basin and the incidence of kidney infection by this myxosporean in smolts collected from the Klamath River and estuary range from 80 – 100% (Foott et al. 1999 & 2002). Impairment of kidney function would be another stressor for smolts attempting to resist other parasitic and bacterial pathogens. This myxosporean may be *Parvicapsula minibicornis* as described by St-Hilaire et al. (2002). No spore stage has been observed to date however, future PCR analysis may aid in its identification.

The relationship observed in this study between elevated water temperature and a rapid decline in gill ATPase activity mirrors other studies (Zaugg & McLain 1976, Duston et al. 1991, McCormick et al. 1999). Further research needs to be done to examine whether smolts have the ability to reverse temperature impairment of smolt development if they later find cooler waters (e.g. estuary, thermal refugia).

Klamath R. steelhead were completely resistant to ceratomyxosis when challenged and reared at various water temperatures. This salmonid population would appear to be at low risk for ceratomyxosis when rearing or migrating in the Klamath River. Disease resistance of the listed Klamath R. coho salmon is not currently known. One moribund coho salmon (40 mm FL) was recovered at the mouth of Pecwan creek (rkm 40) in July 2001 and demonstrated *C.shasta* trophozoites in histological sections (Foott et al. 2002). Udey et al. (1975) reported that juvenile Coho salmon demonstrated a temperature-related ability to defend themselves against lethal ceratomyxosis following a 3 d exposure to infectious water. The authors report that a 201 d cumulative percent mortality

was 84% at 20.5°C and only 22% at 15.0°C. In our study, similar water temperatures did not produce such different mortality patterns in Chinook salmon.

In-situ challenge was used in the current study to allow normal defense mechanisms to operate in the exposed fish. One drawback with in-situ *C.shasta* challenge is the inability to measured or control titer (dosage) of the infectious actinospore contacting the test fish. In an attempt to conduct a standardized challenge and remove seasonal constraints, Ibarra et al. (1992b) employed intraperitoneal injection of cryopreserved *C. shasta* parasites as their challenge method. We were unsuccessful in repeating their cryopreservation and challenge method with *C.shasta* trophozoites and pre-sporogonic stages obtained from Sacramento River adult chinook salmon. Oral gavage and anal intubation of both cyropreserved and fresh material was also unsuccessful in disease transmission in juvenile chinook. Intraperitoneal injection of fresh parasites did result in transmission to salmon and caused typical peritonitis. Yearling steelhead (Sacramento R. strain) were completely resistant to intraperitoneal challenge.

Our hypothesis that chronic exposure to elevated water temperature would suppress non-specific (innate) defenses and thereby increase susceptibility to ceratomyxosis, was not demonstrated by the control salmon held at 20°C. It has been established that physiological responses to short-term stress can stimulate non-specific defenses (Ruis and Bayne 1997). Conversely, chronic stress can impair the immune system and increased disease susceptibility (Peters and Schwarzer 1985, Maule et al. 1989, Schreck 1996, Pickering and Duston 1983). Chronic stress is reported to reduce the alternative complement pathway (ACP) activity of sea bream sera (Tort et al. 1996). In our study, the hemolytic activity of the ACP was not significantly different between salmon reared at 16° compared to 20°C. Cortisol, a steroid hormone central to the stress response, induces apoptosis (host directed cell death) in carp lymphocytes (Verburg-van Kemenade et al. 1999), macrophage proliferation and oxidative metabolism during phagocytosis (Stave & Roberson 1985, Pagniello et al. 2002), and reduces plasma IgM levels in trout (Hou et al 1999). Many physiological responses in cold-water fishes occur to the same magnitude but at faster rates when the fish are held at elevated temperatures. Barton and Schreck (1987) report that juvenile chinook salmon held at 21°C had a faster response to stress but achieved similar plasma cortisol levels as cohorts held at 7.5 and 12.5°C. Trinity R. hatchery fall-run chinook salmon juveniles reared for 14 d at 22° - 23°C had similar plasma cortisol levels as salmon reared at 16° - 17°C (unpublished results, 2001 study, CA-NV Fish Health Center). Sohnle and Chusid (1983) describe how the production of reactive oxygen intermediates by stimulated trout neutrophils occurs faster at elevated temperatures. The complex relationship between environment (temperature), stress response, immune defense, and pathogen virulence will limit most generalizations about fish health and water temperature.

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