U.S. Fish & Wildlife Service

California Nevada Fish Health Center - FY2013 Technical Report:
Pilot Study of the Effects of Saltwater Rearing on C*eratomyxa shasta* Infections in Klamath River Juvenile Chinook salmon, June-August 2013.

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Summary:
Iron Gate Hatchery juvenile Fall Chinook salmon were exposed to Klamath River water for three exposure periods of 24, 48 and 72 hours on June 9-12, 2013 at a site above Beaver Creek. Fish were held at the California-Nevada Fish Health Center wet lab in 18°C ozone treated freshwater for two weeks post river exposure. A sub-sample of the three freshwater exposure groups was assayed by *Ceratomyxa shasta* Quantitative Polymerase Chain Reaction (QPCR) assay and histology just prior to transitioning paired groups to saltwater. Half of the fish from each exposure group were gradually transitioned to full 30ppt saltwater: two days at 10ppt, 2 days at 20ppt and then remained in 30ppt for two weeks.

Exposure groups were monitored for clinical ceratomyxosis by QPCR and histology while held in freshwater, and at the end of the 2 week saltwater rearing period. All freshwater and saltwater mortality was assayed by QPCR to determine cause of death, parasite prevalence and infection level. Following two-week rearing in saltwater, all surviving fish from paired freshwater and saltwater groups were euthanized and sub-sampled for *C. shasta* prevalence of infection (POI) and level of parasite infection measured as *C. shasta* DNA copy number.

This pilot study was conducted to:

- Determine the effects of saltwater environment on various infection levels of *C. shasta* and the progression of ceratomyxosis in sentinel exposed juvenile Chinook salmon, held at 18°C.
- Compare parasite copy number, development of clinical ceratomyxosis and mortality rates in paired freshwater and saltwater groups of juvenile Chinook at three exposure periods.
- Assess whether a larger scale study of saltwater effects on *C. shasta* infection and disease progression is feasible and warrants additional study.

Environmental conditions in the Klamath River above Beaver Creek in mid-June produced low-level *C. shasta* infections in three sentinel groups exposed for 24, 48 and 72 hours. While mean parasite numbers in the intestine of the exposure fish, determined by *C. shasta* DNA copy number, showed a relationship to exposure period/dose, the majority of fish had low infection levels that failed to progress to clinical disease or elevated mortality in fish held for two weeks in pathogen-free water at 18°C. Cumulative Percent Mortality (CPM) in freshwater groups was low during the initial two week period post-exposure (3.9%) and similarly low during the three and four week period post-exposure (9.8%). Total CPM in the three exposure groups reared in freshwater for the 4 week study period was 4.4%. No mortality occurred in the freshwater non-exposure control (NEC) fish.

In saltwater groups CPM during the two-week rearing period was 12.3% in the three exposure groups, and 14% in the NEC group. The majority of Chinook mortality in saltwater groups occurred during the first 48 hours fish were held at 30ppt, indicating a proportion of the Chinook salmon were not able to adapt to full 30ppt saltwater. The similar mortality between exposure groups and the NEC group in saltwater, as well as the timing of mortality, indicates the higher mortality is saltwater groups compared to paired freshwater groups was mainly attributable to inadequate osmoregulatory changes needed to survive in a full concentration seawater environment. However in fish that did tolerate the saltwater environment, we noted *C. shasta* POI decreased over the two-week rearing period,

from 90% in both the 48 and 72-hr groups, to 47 and 43% respectively. The decrease in freshwater groups, held for the same period was markedly lower: from 80-90% in all groups approximately 2 weeks post-exposure to zero in the 48hr group, 13% in the 48hr group and 10% in the 72hr exposure groups at the end of the study (3-4 weeks post-exposure).

Saltwater groups also had reduced *C. shasta* DNA levels, but the decrease in freshwater groups was markedly lower with no detection in the 24hr group, less than 10 copies in the 48hr and 72 hour groups. Saltwater fish were also negative in the 24hr group, and still quite low in terms of DNA levels in the 48hr (17 copies) and 72hr (51 copies) exposure groups.

Additional disease monitoring studies conducted in this part of the basin indicate *C. shasta* infectivity for juvenile Chinook was low in June 2013. In the Klamath river Fish Health Monitoring Study*, Ceratomyxa shasta* prevalence of infection (POI) in Iron Gate hatchery juvenile Chinook captured after residing 1-4 weeks at large (WAL) was 2% (fish were released May 22 – June 5). The highest *C. shasta* POI observed in the Shasta to Scott river reach, which includes the Beaver Creek exposure site, was 10%. Mean DNA copy number for all juvenile Chinook salmon captured in this reach was very low at 30 copies. Sentinel studies conducted by Oregon State University at Beaver Creek in May and June also showed low mortality rates for juvenile Chinook salmon. Seventy-two hour sentinel exposures of IGH Chinook in May had no mortality and three groups exposed in June had an overall *C. shasta* loss of 5.2% when held at 18°C post-exposure (Hallett 2013). Other evidence from OSU water monitoring studies at the Beaver Creek site demonstrated actinospore density did not surpass 10 spores/L in June.

In summary, IGH juvenile Chinook salmon exposed for 24, 48 and 72 hours above Beaver Creek developed low level infections of *C. shasta* that resolved over 4 weeks when fish were held in pathogen-free freshwater, and decreased to a lesser degree in fish transitioned to saltwater, compared to their freshwater cohorts. *C. shasta* CPM, POI and DNA copy number all showed a dose exposure response, but with low level infection and little variation in infection levels between the three exposure periods.

In conclusion, data from this pilot saltwater study and additional sentinel and monitoring studies in this area of the basin indicate infectious conditions in the Klamath River in June were low compared to previous years. The low infectivity of river water in June, and resulting low infection levels in all three exposure period groups, did not allow adequate infection in juvenile Chinook to fully assess the effects of saltwater environment on *C. shasta* infection and disease progression.

Introduction:

Annual prevalence of *Ceratomyxa shasta* infection in out-migrating juvenile Chinook salmon in the Klamath River has been well studied for over a decade (Hendrickson et al. 1989, Foott et al. 1999, True et al. 2013). Route of infection (Bjork & Bartholomew 2010), disease susceptibility and prognosis (Foott et al. 2004, True et al. 2011) and disease dynamics related to environmental conditions have been well researched (Ray et al 2012, Hallett et al. 2012). These studies describe a relationship of actinospore exposure within the infectious zone (Shasta Dam to Seiad Valley) in late Spring and early Summer in both natural fish and Iron Gate Hatchery Chinook salmon releases that generally occur in late May or early June. In monitoring studies, *C. shasta* infections generally progress to clinical ceratomyxosis, over a 7-18 day period, depending on exposure dose and period fish experience during out-migration through the infectious zone. The development of disease is largely driven by overall exposure dose and river temperatures. Lower river temperatures (Udey et al. 1975) can prolong disease development to a degree, however once an infection threshold is reached, lower temperature is no longer a mitigating environmental factor (Ray 2009). Sentinel studies conducted by Oregon State University (Hallett, BOR reports and personnel communication) suggest water born exposures of 10 actinospores/L result in significant mortality rate in susceptible Rainbow trout, Chinook and coho juvenile salmon.

The primary objective of this study was to determine the relationship between the prevalence and severity of *Ceratomyxa shasta* infection acquired by juvenile Chinook salmon in freshwater to the subsequent prevalence and severity of ceratomyxosis following smoltification and entry in saltwater occurs.

Methods:

Experimental Fish

Approximately three-hundred juvenile Fall Chinook salmon obtained from Iron Gate Hatchery on June 3 were transported to the Ca-Nv FHC wet laboratory and acclimated to 18°C pathogen-free water for 7 days prior to sentinel exposure on June 10. Experimental fish were held in 800L circular tank supplied with 7.6 L/min flow of ozonated water and fed a BioMoist #1 diet at 3% body weight pre-exposure and ad lib post-exposure. Oxytetracycline medicated feed was prophylactically fed at rate of 5g/100 pounds of fish for 3 days prior to exposure periods and 3 days post-exposure, to preclude *Flavobacterium columnare* infections which are frequently contracted by sentinel fish at the Beaver Creek exposure site. Fish were taken off food 24 hours prior to river exposure, and given prophylactic Furanase treatments (1mg L^{-1}) during the 1.5 hour transportation time to and from the exposure site. Hobo Data loggers (Onset Corporation, Pocasset Maine) were used to monitor water temperatures in the transport tank, in live cages during the 72-hour exposure, and in the wet lab holding tanks during acclimation and post-exposure periods.

Pre-screening by QPCR for pre-existing myxozoan infections

Thirty fish were tested by QPCR prior to river exposure, to ensure no pre-existing infections of *C. shasta* were present. Entire intestine were removed, extracted for total DNA, and assayed for each myxozoan by the QPCR methods described below.

Non-Exposed Control Groups

Chinook salmon were counted and 60 fish were randomly netted and assigned to each exposure period group and every fifth fish, for a total of 200 Chinook, was assigned to a

Non-Exposed Control (NEC) group.

24, 48 and 72-hour Sentinel Exposures

The exposure site lies within the "infectious zone" of the Klamath River, at RM 163, which is approximately 1 mile above the confluence of Beaver Creek (UTM 10 516058E 4634926N) (Figure 1) .

Thirty fish from each assigned exposure period group were randomly netted and placed in one of two replicate 0.34ft^3 live cages (total of 6 live cages). Live cages were transported within a larger 1000gal transport tank to the Klamath River on June 10, 2013. Live cages were placed approximately 4 feet from the bank at the river bottom. After each 24 hour period, two of the live cages specific to the exposure period, total of 60 fish, were transported back the Center's wet lab and returned to 800L circulars. Fish were held in freshwater at 18°C for two weeks.

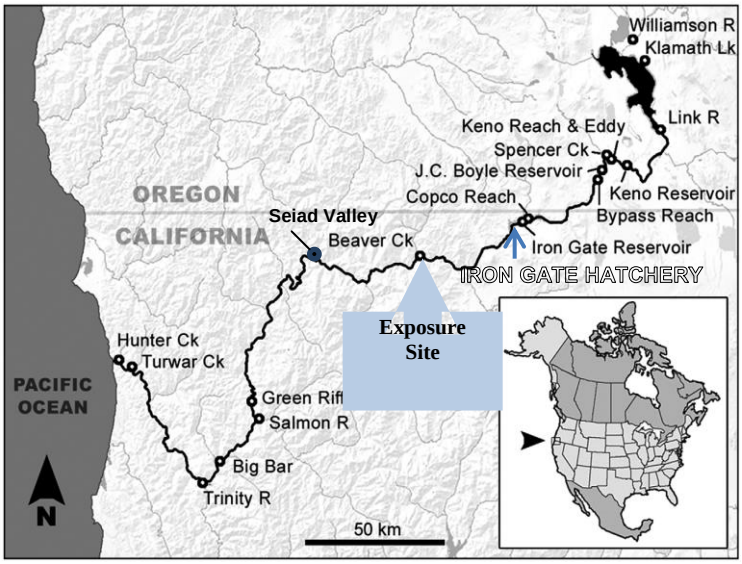

Figure 1. Exposure site above Beaver Creek (RM 163) on the Klamath River in northern California. The "infectious zone" extends from Iron Gate Dam to Seiad Valley (RM 141 to 190).

All exposed fish and NEC groups, resumed feeding 24 hours post-exposure and were monitored for mortality for four weeks. All fresh mortality was sampled by QPCR to determine cause of death and *C. shasta* infection level, if present.

Water Sampling to Estimate Exposure Dose

An ISCO automated water sampler (Teledyne Isco, Inc. Lincoln, NE) was used to collect 2L water samples every 2 hours for a composite water sample for each exposure period (24hr, 48hr and 72hr). Water samples were kept on ice, vacuum filtered and DNA extracted using Qiagen DNeasy™ kits (Qiagen, Valencia CA) following the manufacturer's protocol for animal tissues. Specifically, filter membranes were folded into individual sterile, 2 mL microcentrifuge tubes. Filters were lysed with double volumes of ATL lysis buffer (360uL) and Proteinase K (40uL) in order to full immerse filters during this step of extraction. Purified DNA was eluted in a single 200uL volume of elution buffer and frozen at -20°C until assayed. QPCR (Hallett 2006) was used to determine DNA copy number/L of water and estimated actinospore dose for each exposure period. While the Cs QPCR cannot distinguish actinospores from myxospores in water samples, test results are presumed to be primarily actinospores.

Mortality Monitoring

The three exposure groups of 24, 48 and 72 hours were intended to provide various infection levels in juvenile Chinook salmon that could be compared in paired freshwater (FW) and saltwater (SW) rearing groups. All fresh mortality that occurred in the initial freshwater groups was placed in 6 oz. individual zip-lok™ bags, and frozen at -20°C. Prior to transition to saltwater, a sub-sample of 10 fish from each exposure period and 20 fish from the NEC was tested by QPCR and histology to determine pre-saltwater infection levels (DNA copy number). All surviving fish from freshwater and saltwater groups were necropsied at the conclusion of the study and tested by QPCR.

Cumulative Percent Mortality was calculated for each exposure group held in freshwater, and for fish transitioned to saltwater two weeks later on 26 June. Percent Mortality equals the sum total of mortality during total holding period, divided by the total mortality in each specific group (x 100). Mean Day to Death (MDD) for each exposure group was calculated as the geometric mean of all days with *C. shasta* mortality within the 28 DPE post-exposure holding period.

Histology

A composite pathology score was determined for each fish sampled. Intestine was rated for quantity of *C. shasta* trophozoites (0=none, 1= 1-10 per section, >10 per section) and presence of myxospores. The relative percentage of the epithelium that was hemorrhagic, eroded, or necrotic was recorded for each section. We arbitrarily selected 50% of the section as a threshold value for severe clinical ceratomyxosis: 50% erosion, hemorrhage and necrosis (EHN).

Saltwater Rearing

On June 26, which corresponded to 13-16 days post exposure (DPE) depending on the specific exposure group, half of the fish from each exposure period were moved to 1.5 ft3 individual cages within a larger tank. Fish were transitioned to 10ppt saltwater water by slowing adding Instant Ocean™ salt granules and mixing thoroughly. Salinity was checked at each saltwater concentration using a refractometer at the onset of the period and every 24 hours. Additional salt was added at 48 hours to bring saltwater concentration up to 20ppt, and again at 72 hours for a final concentration of 30ppt. Fifty percent water changes were performed every three days during the extended rearing period: supplemental Instant Ocean™ was added at each water change to maintain the 30ppt salt concentration. Fish were held in this concentration for two weeks, until 9 July, when the study was terminated and all freshwater and saltwater fish were necropsied and assayed for *C. shasta* by QPCR and histology.

Table 1. Distribution of exposed and NEC juvenile Chinook salmon in freshwater and saltwater phases of the study.

Experimental Fish - Acclimation, Exposure and Rearing Groups		
Acclimation and Pre-screening	**Freshwater Rearing**	**Saltwater Rearing**
No. Fish Pre-screened by QPCR (June 5)	30	NA
No. Fish assigned to Non-Exposed Control (NEC) Groups: FW-NEC (Initial group of 200) SW-NEC (Moved to SW June 25)	150	50
Exposure Groups		
24 hour exposure (June 10-11) No. Mortality during initial FW rearing No. Fish tested prior to SW No. Fish Maintained in FW No. Moved to SW (June 25)	60 1 10 24	25
48 hour exposure (June 10-12) No. Mortality during initial FW rearing No. Fish tested prior to SW No. Fish Maintained in FW No. Moved to SW (June 25)	60 2 10 23	25
72 hour exposure (June 10-13) No. Mortality during initial FW rearing No. Fish tested prior to SW No. Fish Maintained in FW No. Moved to SW (June 25)	60 4 10 23	23

QPCR Assays for *C. shasta* Infections in Fish

At the conclusion of the four week study, survivors from both freshwater and saltwater groups were euthanized and necropsied for *C. shasta* by QPCR and histology.

A DNA extraction control derived from naturally infected fish tissues, and confirmed by histology, was standardized to a known C_T value and Cs DNA copy number. The extraction control was included for each set of samples processed to monitor extraction efficiency and preclude false negative assay results which can arise from poor quality or low quantity of total DNA.

Quantitative PCR for *C. shasta* (Hallett 2006) in fish tissues was performed in a final volume of 30μL containing 1x Universal master mix (Applied Biosystems), 900nM concentration of each primer, 250nM concentration Taqman® MGB Probe and 5uL DNA template (approximately 300ng / reaction). Absolute standards (described below), no template control (NTC) wells consisting of molecular grade water, and an extraction control were included on each assay plate. Reactions were analyzed with an Applied Biosystems 7300 Sequence Detection System® (SDS) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40-45 cycles of 95°C for 15s and 60°C for 1 min. Assay threshold was verified for each run and set manually to 0.20 using the SDS software (v1.3.1, Applied Biosystems).

Plasmid DNA controls and Standard Curves for Water Samples

C. shasta actinospore and myxospore exposure dose L^{-1} (spore concentration) was calculated using a standard curve developed by Oregon State University (OSU) for sentinel studies conducted at various locations in the Klamath basin (Hallett and Bartholomew 2006). The slope of the *C. shasta* QPCR standard curve is -4.0166 with a y-intercept of 34.7. The amplification efficiency (E) of synthetic template DNA was calculated to be 83% using the formula *E = 10–1/s – 1 (Applied Biosystems Guide to Quantitative Gene Expression). Ceratomyxa shasta* plasmid standards with known parasite copy number, NTC and extraction controls were run on each water sample plate. Water sample C_T values were transformed to spore L-1 concentrations by the method of Hallett and Bartholomew (2006).

To preclude QPCR assay inhibition in environmental samples, water samples were tested at three dilutions (1:10, 1:100, and 1:1000). The average Cs spore L^{-1} was calculated for the composite samples for the 24, 48 and 72 hour exposure periods.

All survivors from the freshwater and saltwater groups, as well as tissues from frozen mortalities from each rearing environment and exposure group, were tested by QPCR to determine cause of death, *C. shasta* POI and DNA copy number for each experimental group.

Results and Discussion:

Pre-screening by QPCR for pre-existing myxozoan infections

IGH Chinook salmon tested negative for *C. shasta* by QPCR prior to river exposure.

Exposure Dose

Water samples tested by QPCR determined the mean *C. shasta* spore density for 72 hour exposure period to be 1.47 spores/L. Standard deviation (1.0) and spore concentrations were low for the three exposure periods of 24 hour (1.1), 48 hour (0.64) and 72 hours (2.6) : all three exposure days had levels below the 10 spore/L threshold that results in moderate to high mortality in juvenile salmonids held at 18°C post-exposure.

CPM in FW Groups:

Cumulative percent mortality was low during the first two weeks post-exposure in the three exposure groups (Figure 2): mortality rates followed expected exposure period with the lowest CPM in the 24hr group (1.7%) followed by the 48hr group (3.3%) and the highest CPM in the 72hr group (6.7%). During the 3rd and 4th weeks post-exposure, rates were higher in both the 24hr group (4.2%) and the 72hr group (13%), as infections progressed.
No additional mortality occurred in the 48hr group at 14-28 DPE, and the NEC groups had no mortality throughout the same period. Over the entire 4 week rearing period, total CPM in freshwater groups was 3.3% in both the 24hr and 48hr exposure groups, and 13% in the 72hr group. The 72hr group appeared to have received sufficient actinospore exposure to cause low level mortality. However we did not observe clinical disease during rearing or the elevated mortality generally observed in 72hr sentinel studies conducted at this site (with similar post-exposure holding at 18°C).

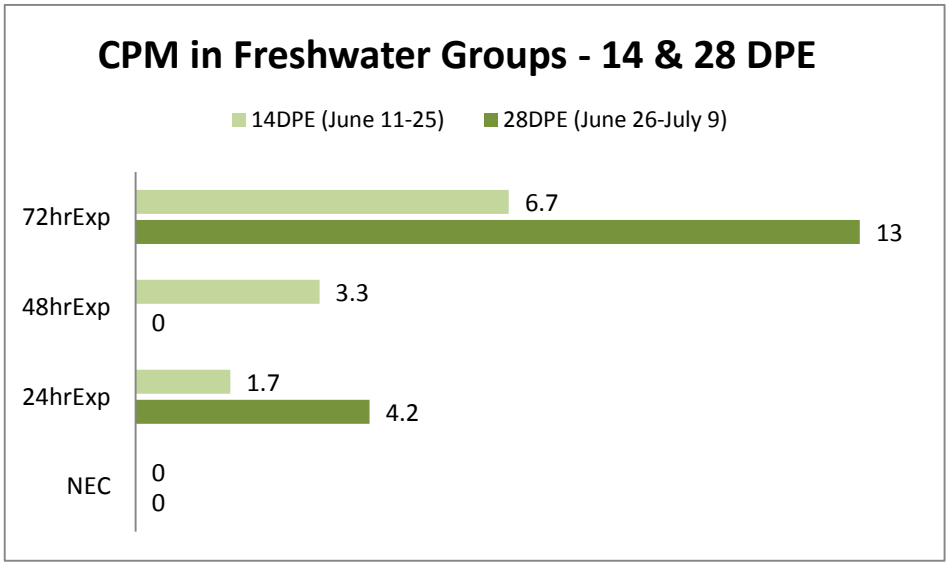

Figure 2- Cumulative percent mortality in freshwater groups post-exposure. Initial CPM for the first two weeks post-exposure (June 11-25) when all fish were held in freshwater. CPM in FW groups during the following two week period (June 26-July 9) which coincides with paired groups held in saltwater.

Ceratomyxa shasta Infection Levels by QPCR and Histology

Sub-samples were collected 25 June for QPCR and histology, prior to SW transition, to assess infection levels and parasite numbers in the intestine of fish reared in freshwater. Upon necropsy, early clinical signs of ceratomyxosis were observed in all groups, but were markedly less severe in the 24hr exposure group. In the 48 and 72hr groups, the clinical disease score of +1 was assigned to the majority of fish due to hemorrhaging of the primary intestinal artery, as well as early hemorrhaging of the lower intestine (pink to light red coloration of the intestinal wall/epithelium) (Figure 3). In the 24hr group, the clinical presentation was limited to the upper intestine where yellow exudate was observed; no hemorrhaging of the lower intestine was observed in this group.

Figure 3. Clinical disease signs developing in the 72hr exposure groups at 14 DPE (25 June) prior to transition to saltwater. Anterior intestine with some yellow exudate present. Posterior intestine shows inflammation and mild hemorrhaging.

Histological results were negative for *C. shasta* trophozoites in the intestine, in both the pre-saltwater samples and in the fish screened at the conclusion of the study. The only conclusion that can be drawn is that *C. shasta* infection levels were below the detectable threshold of this screening method, before fish entered saltwater two weeks post exposure and at the conclusion of the study. QPCR data supports this finding, as levels by this highly sensitive methodology were also very low at both sampling events. Therefore, QPCR data was the only method available in this pilot study to evaluate and quantify *C. shasta* infection levels or progression of disease following saltwater rearing.

Moderate infections of *Parvicapsula minibicornis* were detected in 100% of kidney tissues tested by histology, except the non-exposure control group, in both pre-saltwater and study termination samples. No kidney lesions were observed by histology that would indicate a problem with saltwater transition due to parasite infection. In the Klamath River, early onset and high prevalence of infection with *Parvicapsula minibicornis* is common in juvenile Chinook salmon, and generally does not cause clinical disease (glomerular nephritis).

All fish appeared equally developed in terms of a ranked smoltification index (silver coloration and scale loss) prior to saltwater rearing (Figure 4).

Figure 4. Silver smolt coloration and minor scale loss observed in all fish sampled from freshwater exposure groups on 25 June, prior to saltwater transition.

CPM in Saltwater Groups:

Following transition to 30ppt saltwater, the CPM increased in all saltwater rearing groups during weeks 3-4 post-exposure, including the NEC, compared to their freshwater cohort groups (Figure 3). Mortality rates were highest in the 72hr group, and followed a dose relationship with the exception of the NEC group at 14%. The majority of the mortality occurred within 48 hours of entering full strength saltwater. The data indicate at least a proportion of the mortality in the saltwater groups is attributable to an inability of some fish to physiologically adapt to full saltwater NaCl concentration of 30ppt. In the longest exposure (72hr) group, the difference in CPM was approximately 3% higher in the saltwater group compared to the group held in freshwater. However the difference is higher in the NEC fish (14%) and 48hr group (12%), again indicated non-specific mortality attributable to the saltwater environment.

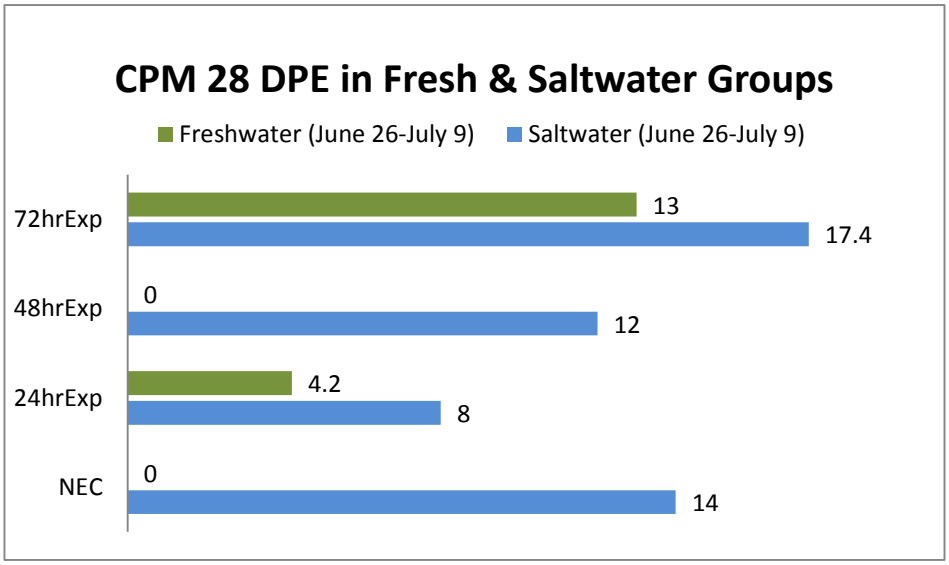

Figure 5- Cumulative Percent Mortality in Fresh and Saltwater Groups, during the same post-exposure period of June 26- July 9 (14-28 DPE).

Interestingly, *C. shasta* POI was relatively high (80-90%) in freshwater groups at 14 DPE, even though CT values indicated infection levels were relatively low (Figure 6). Prevalence of infection dropped markedly in the freshwater groups during the 3-4 week period post-exposure, especially when compared to their saltwater cohorts. At the end of the two week saltwater rearing period, *C. shasta* POI was 11, 47 and 43% in the 24hr, 48hr and 72hr groups (respectively) compared to zero, 13 and 10% in the freshwater groups. In addition to increased mortality in the saltwater groups discussed above, juvenile Chinook held in saltwater were slower to resolve *C. shasta* infections, compared to their freshwater cohorts. This could be due to additional energy and physiological demands of saltwater rearing. Also apparent in this data was how similar the 48 and 72hr groups appeared to be in their disease response (POI), despite different exposure periods. Both groups had similar freshwater *C. shasta* POI at 14 DPE, as well as similar POIs during both the freshwater and saltwater rearing of 3-4 weeks post-exposure. CT values were somewhat lower (more parasite DNA) in all 72hr groups compared to the 48hr groups.

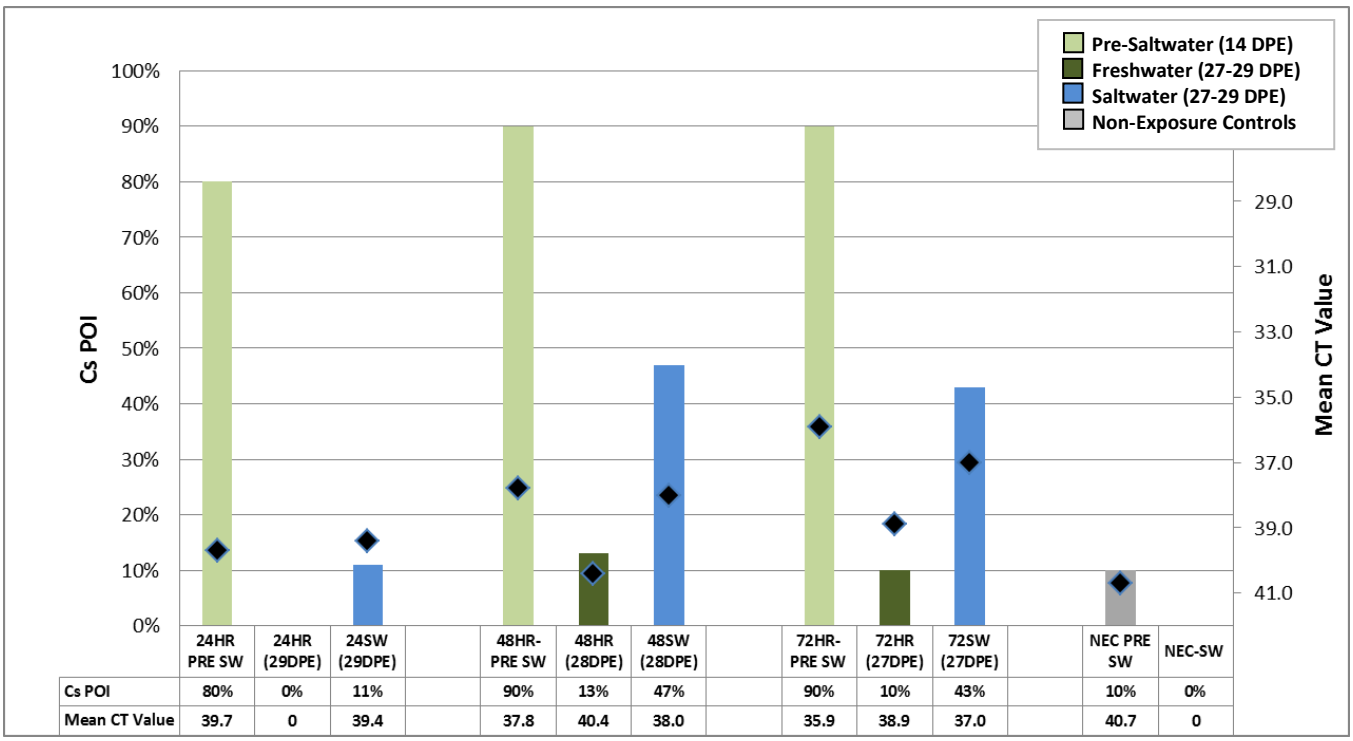

Figure 6. Ceratomyxa shasta Prevalence of Infection (POI) and Mean CT Value in Freshwater, Saltwater and Non-Exposure Controls.

Ceratomyxa shasta DNA copy number at 14 DPE (prior to saltwater transition) corresponded well to exposure periods, in that the lowest infection levels were observed in the 24hr exposure group, and levels increased in relation to exposure period. While an increasing trend in DNA copy number was observed in the exposure groups, the levels of *C. shasta* DNA overall was relatively low in all groups. In the annual KR fish health monitoring studies, clinically diseased fish sampled two to three weeks post hatchery release often carry DNA copy numbers in broad, but higher ranges of 3-7 logs (True et al. 2011, True et al. 2012, and Bolick et al. 2013 – *in preparation*).

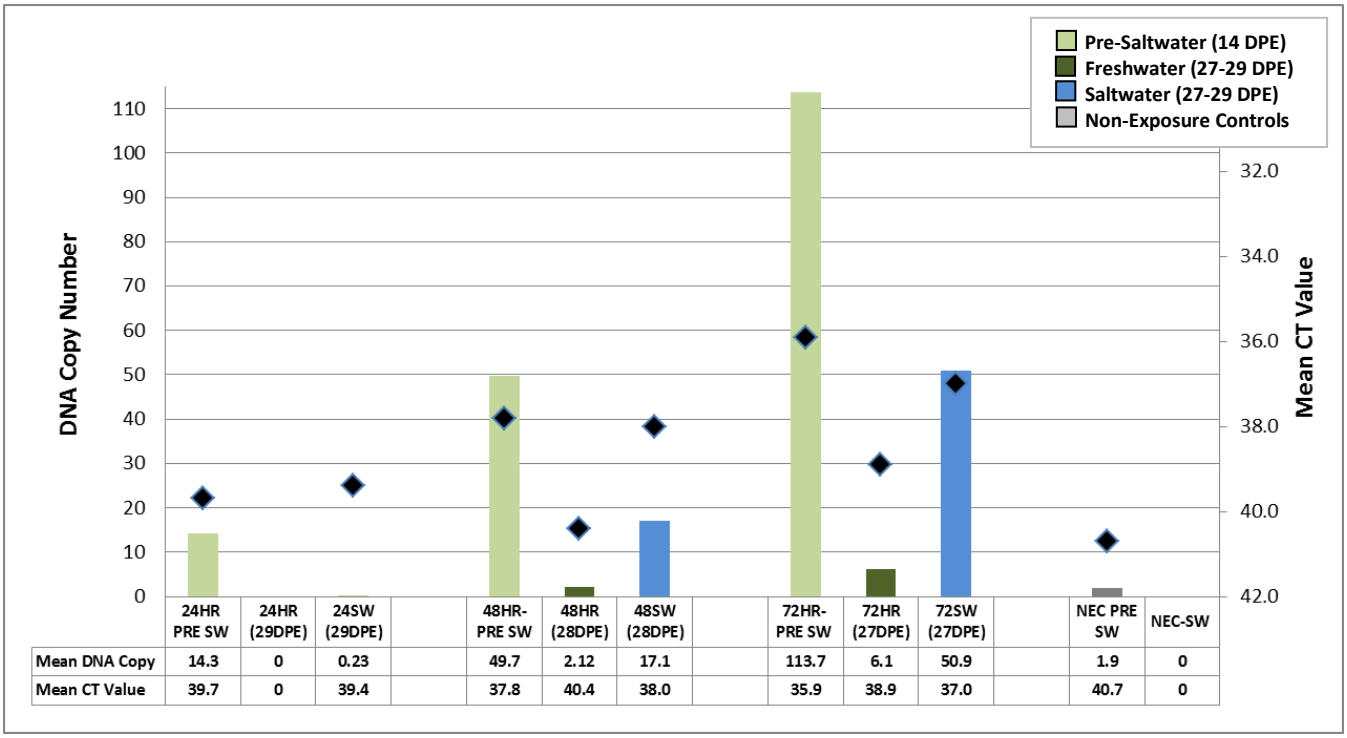

Figure 7. *Ceratomyxa shasta* mean DNA copy number and mean CT value in freshwater, saltwater and non-exposure controls.

<u>Parasite Levels in Mortalities</u>

As noted earlier, the majority of mortality in the SW groups occurred at 48-72 hours after entering 30ppt saltwater (July 1-2). However a few of the mortality fish had notable *C. shasta* infection levels by QPCR, particularly in the 72hr exposure groups (Table 2).

In the 72hr FW groups, 1/6 fish tested had over 3800 DNA copies present in the intestine at 12 DPE. In the 72hr SW group, two fish had elevated levels of *C. shasta* DNA at 890 and 91,785 copies at 19-21 DPE. Both of these SW rearing fish had noted hemorrhaging of the intestine on necropsy. However the other two morts observed in this group had low levels of *C. shasta* parasite not likely to have caused mortality. Mean CT values for 72hr FW was 34.5 compared to 33.5 for the 72hr SW group.

QPCR testing of mortality in the SW-NEC group confirmed these fish died of osmoregulatory problems rather than *C. shasta* infections, as all fish tested negative. Overall, there appeared to be a mix of clinically infected fish in the 72hr exposure group, and some mortality related to the saltwater transition. Feed was offered to all fish held in saltwater, but appetence was minimal during the transition period. Fish appeared hungry and took food approximately a week after entering 30ppt saltwater. So malnutrition may have played a role, although likely minor, in the observed mortality in fish with low level infections.

Table 2. Infection Levels in Mortality Fish – All Groups. Fish ID, Date, DPE, rearing salinity (PPT), clinical signs, mean CT and DNA copy number for all mortality fish in the study.

Fish ID	Mortality Date	Days Post Exposure	Salinity (PPT)	Clinical signs on Necropsy	Mean CT	DNA Copy Number
24HRFW-1	**6/16/2013**	6	NA		36.4	42
24HRFW-2	**7/8/2013**	27	NA		NEG	NA
48HRFW-1	**6/16/2013**	5	NA		38.0	12
48HRFW-2	**6/19/2013**	8	NA	Very Necrotic - poor quality tissue	40.1	3
72HRFW-1	**6/19/2013**	7	NA		37.3	19
72HRFW-2	**6/24/2013**	12	NA	Hemorrhaged IT	29.1	3830
72HRFW-3	**6/24/2013**	12	NA	Fish too necrotic - Not Tested	NA	NA
72HRFW-4	**6/24/2013**	12	NA		32.3	469
72HRFW-5	**6/27/2013**	15	NA		33.0	313
72HRFW-6	**6/28/2013**	16	NA		37.6	58
72HRFW-7	**7/6/2013**	24	NA		37.5	60
24HRSW-1	**7/1/2013**	19	30	Hemorrhaged IT	34.8	333
24HRSW-2	**7/2/2013**	20	30		37.1	77
48HRSW-1	**7/1/2013**	20	30		37.8	51
48HRSW-2	**7/2/2013**	21	30		39.1	23
48HRSW-3	**7/2/2013**	21	30		39.0	24
72HRSW-1	**7/1/2013**	19	30	Hemorrhaged IT	25.7	91785
72HRSW-2	**7/2/2013**	20	30		39.5	18
72HRSW-3	**7/3/2013**	21	30		35.6	200
72HRSW-4	**7/3/2013**	21	30	Hemorrhaged IT	33.2	890
NEC-SW-1	**7/1/2013**	NA	30		NEG	NA
NEC-SW-2	**7/1/2013**	NA	30		NEG	NA
NEC-SW-3	**7/1/2013**	NA	30		NEG	NA
NEC-SW-4	**7/2/2013**	NA	30		NEG	NA
NEC-SW-5	**7/2/2013**	NA	30		NEG	NA
NEC-SW-6	**7/2/2013**	NA	30		NEG	NA

Study Survivors

At the termination of the study, which was 27-29 DPE depending on the specific exposure group, all surviving fish were assayed by QPCR (Table 2). In all exposure groups transitioned to saltwater, the mean CT value was lower (CT values are inverse, lower CT indicates more *C. shasta* DNA) than in the paired freshwater groups. The mean CT value in the saltwater groups was approximately 2 CT lower than the freshwater cohort group, indicating a ~10-fold higher concentration of *C. shasta* DNA after 14 days in a saltwater environment.

Table 3. Study Survivors: *Ceratomyxa shasta* prevalence of infection (POI), mean CT, CT Range, and Mean DNA Copy Number at the termination of the study. Note: DPE varies by 1-3 days for each exposure group, all saltwater groups were held for the same period (14 days) in saltwater, which includes the transition period of 4 days from 10ppt to 30ppt.

Exposure Group	DPE	Days in SW	*Cs Positive*	N	Cs POI	Mean CT Value	CT Value Range	Mean Copy Number
24FW	29	0	0	23	**0%**	**NA**	NA	**0**
24SW	29	14	2	18	**11%**	**39.4**	**39.2 & 39.7**	**0.23**
48FW	28	0	3	23	**13%**	**40.4**	**39.6-41.0**	**2.12**
48SW	28	14	8	17	**47%**	**38.0**	**35.6-41.0**	**17.1**
72FW	27	0	2	20	**10%**	**38.9**	**38.2 & 39.6**	**6.1**
72SW	27	14	6	14	**43%**	**37.0**	**33.8-40.3**	**50.9**

To summarize, the highest *C. shasta* POI (47%) occurred in the 48hr exposure group, reared in saltwater at 28 DPE, but was similar to the value observed in the 72hr saltwater group (43%). The highest quantity of *C. shasta* DNA was detected in the 72hr group held in saltwater, with a mean of 51 copies. Chinook salmon in both the 48 and 72hr freshwater groups were infected with *C. shasta* with POI of 13% and 10%, respectively. However, these low infection prevalences, as well as low DNA copy numbers, indicate these exposure groups had infection levels that were not likely to lead to significant mortality rates (for example, mortality >50% for fish held ~21 DPE at 18°C is not unusual for sentinel studies at this site in June).

Conclusions:

Mortality and *C. shasta* QPCR data indicate juvenile Chinook salmon become infected when exposed to Klamath River water for 24, 48 and 72 hour periods. However infectivity at the sentinel study site above Beaver Creek did not result in significant mortality or *C. shasta* infection levels as screened by both QPCR and histology. One objective of the study was to produce 3 increasing infection levels in juvenile Chinook salmon that could be used to assess the effects of saltwater rearing on infection level and progression of clinical ceratomyxosis.

While the data did suggest a dose response for increasing exposure periods (in terms of *C. shasta* mortality, POI, and DNA copy number), all groups had relatively low and similar infection levels by QPCR. Negative histological screening confirmed this as well. In fact, infection levels were so low, they resolved in the shortest exposure period group (24 hours) within 4 weeks post-exposure when fish were reared on pathogen-free freshwater. In the longer exposure period groups of 48 and 72 hour, there was a marked decrease in *C. shasta* POI from 2 weeks post-exposure (90%) to 4 weeks post-exposure (10-13%) in the freshwater

groups. This suggests that these groups were also in the process of recovering from low-level infections that occurred during sentinel exposure. While saltwater groups did not show the same decrease in *C. shasta* POI as their freshwater cohorts, there was a marked decrease from 80-90% at ~14DPE to 10-47% during the two weeks they reared in a full 30ppt environment (~28DPE). A small proportion of the juvenile Chinook were not well adapted to survive in 30ppt saltwater. The majority of mortality across all the saltwater groups occurred after 48 hours in 30 ppt saltwater, a period when non-adapted fish are likely to die due to dehydration, if unable to osmoregulate adequately. Mortality was also high in the Non-Exposed Control fish in saltwater, which were not infected with *C. shasta*, again indicating some lack of adaptation to a marine environment.

Recovery from low-level *C. shasta* infections has been observed in previous studies, primarily by histology. In juvenile steelhead, trophozoites were only observed in the intestinal lumen (Stone et al. 2006, Foott et al. 2004) and did not penetrate the intestinal epithelium, indicating host resistance to *C. shasta* infections at low infection levels. We have also observed focal granulomas on the serosal surface of the intestine, containing low numbers of myxospores but no pre-sporogonic forms, in the *C. shasta* short exposure studies (3-6 hour) that coincided with resolution of infection (Foott et al. 2006). In a prognosis study with Chinook and coho salmon, a small proportion of fish showed no infections, despite high mortality and infection levels in cohort fish in the same exposure groups (True et al. 2012). This finding likely indicates these fish experienced a lower dose at exposure and were able to fully recover from low level *C. shasta* infection.

The study appears to be worthy of repeating considering how important early ocean survival is in terms of adult contribution to the fishery and subsequent escapement to the basin. Little is known about the fate of *C. shasta* infections as juvenile Chinook salmon enter the estuarine and ocean environments. Intuitively, one would expect the combination of clinical infection and the stress of physiological changes required for saltwater osmoregulation to be taxing energetically. The lethargy observed in clinical ceratomyxosis suggests weak fish would not be able to afford the added expenditure of energy needed to survive the marine environment. But probably more interesting is the prognosis of low to moderately infected fish, as they generally comprise a larger proportion of natural and hatchery juvenile Chinook salmon in any given year. The study showed a dose response in infection levels related to exposure period, and even though infection levels were low in all groups, there was an observed difference in how *C. shasta* POI responded 3-4 weeks post-exposure in the freshwater and saltwater rearing environments. It would be interesting to see at what infection level the *C. shasta* parasite compromises the ability of fish to survive in the marine environment.

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