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Long-Term Fish Disease Monitoring Program in the Lower Klamath River

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This report addresses the actions described in the Supplemental Statement of Work, July 2012 for completion of 2011 project objectives and archiving samples for model development and analysis.

Objective 1: Develop a multiyear dataset on *Ceratomyxa shasta* infection prevalence in both host populations (fish and polychaete) at selected locations to monitor how changes in flow, temperature and other variables alter parasite infection rate.

**Task 1.2 Sentinel fish exposures**

Additional ISCO units and temperature loggers were obtained.

Visual examinations of all fishes to determine *C. shasta* infections have been completed. *Ceratomyxa shasta*-PCR testing of fish (mortalities) that were negative by microscopic examination has been completed. The following graphs and summary have been updated with these additional data.

![Graph showing percent mortality of rainbow trout and IGH fall Chinook salmon exposed April 25-28, 2011 at one sentinel index site in the Klamath River basin and held for 60 days post-exposure at 13°C. KOR=Orleans, KSV=Seiad Valley, KBC=upstream of Beaver Creek, KKB=Klamathon Bridge, KED=Keno Eddy, WMR=Williamson River.](image-url)
Fig. 1.2.2. Percent mortality of rainbow trout, IGH fall Chinook and coho salmon exposed May 17-20, 2011 at six sentinel index sites in the Klamath River basin and held for 60 days post-exposure at 13°C. See Fig. 1.2.1 for site abbreviations.

Fig. 1.2.3. Percent mortality of rainbow trout, IGH fall Chinook and coho salmon exposed June 21-24, 2011 at seven sentinel index sites in the Klamath River basin and held for 60 days post-exposure at 18°C. See Fig. 1.2.1 for site abbreviations; KTC=Tully Creek
Fig. 1.2.4. Percent mortality of rainbow trout and IGH fall Chinook salmon exposed September 23 - 26, 2011 at two sentinel index sites in the Klamath River basin and held for 60 days post-exposure at 18°C. See Fig. 1.2.1 for site abbreviations.

Fig. 1.2.5. Effect of post-exposure water temperature on percent mortality for groups of rainbow trout (80 fish), Iron Gate Hatchery Fall Chinook salmon (80 fish) exposed for 72 hours near Beaver Creek in April (A), May (M), June (J), and September (S) 2011 and for coho (60 fish) in May and June and then divided in half and held at either 13°C or 18°C for 60 days.
Fig. 1.2.6. Comparison of percent loss of juvenile IGH fall Chinook salmon exposed in the Klamath River for 72 hr in June 2007-2011.

Fig. 1.2.7. Comparison of percent loss of juvenile coho salmon exposed in the Klamath River for 72 hr in June 2007-2011. In 2011, coho were held only near Beaver Creek and Seiad Valley.
Summary of the sentinel results:

- Similar to previous years, *C. shasta* infections were detected in susceptible rainbow trout in all months tested in 2011, i.e. April, May, June, and September near Beaver Creek and at all sentinel sites in May and June in the upper and lower Klamath River except at Klamathon in June. However, in 2011 the rainbow trout loss was much lower than 2010 in fish exposed in April near Beaver Creek and in May and June at Keno Eddy and Klamathon. Losses were high at Williamson River and most lower river sites in May and June.

- Compared to sentinel results of 2007-2009, losses of IGH fall Chinook were much lower in both 2010 and 2011. Cooler water temperatures during the spring exposures in 2011 resulted in decreased loss in IGH Chinook for both May and June. However, in June, some loss occurred in Chinook held in the “infectious zone” of Beaver Creek (17%) and Seiad Valley (12%). Highest mortalities followed exposure at the Beaver Creek site, and decreased downstream to Seiad Valley and Orleans. This pattern has been consistent across all years (2007-2011).

- Even though water temperatures near Beaver Creek were low in May, the coho were more affected in 2011 compared to 2010. The IGH coho salmon exposed at Beaver
Creek in May and Beaver Creek and Seiad Valley in June incurred much higher loss; at Beaver Creek (51% at 18°C in May and 53.8% in June) and 60.8% at Seiad Valley in June. In some diseased coho, *C. shasta* was detected only in the eyes. Although no myxospores were detected in the usual target organ (the intestine) in these fish, *C. shasta* was still considered the cause of death.

- The lower Williamson River in the upper Klamath Basin was tested for *C. shasta* in May and June. More than 97% of the rainbow trout died with *C. shasta* infections but no infections were detected in the Chinook.

- Post-exposure rearing water temperature affected percent mortality of fish exposed near Beaver Creek. More Chinook died at 18°C than 13°C, e.g. 17.0% versus 2.5% in June. The effect of temperature was more pronounced for coho salmon, e.g. in May, 6.7% died at 13°C versus 51.6% at 18°C. This difference in response to temperature, i.e. coho are more affected by elevated water temperature than Chinook, has been observed consistently among years.

- Comparison of sentinel study results from 2007, 2008, 2009, 2010 and 2011 for fish exposed near Beaver Creek shows a shift in the level of mortality between coho and Chinook salmon. In 2007, coho loss in May and June was much higher than for Chinook. In 2008, both species incurred very high losses after exposure. In 2009, the loss of Chinook was higher than coho from *C. shasta*. In June 2010, losses in Chinook were again higher than coho, i.e. 20% versus 10%, but mortality for both species was much lower than in previous years. In 2011, coho salmon had much higher loss in May and June compared to the Chinook salmon.

**Task 1.3 Water sample collection and parasite density determination**

Additional students were trained in molecular methods to enable completion of project tasks. The qPCR machine was serviced.

A subset of water samples (*n*=278) was genotyped using the new assay described in Task 3.2. We focused on one index site (Beaver Creek, KBC) across six monitoring years (2006-2011) and sequenced weekly samples when available. We also sequenced all samples available for the other four index sites (Klamathon Bridge, KKB; Seiad Valley, KSV; Orleans, KOR; and Tully Creek, KTC) in two years of high total parasite density, 2007 and 2009. Only samples with sufficient DNA present (determined in the SSU-qPCR as those with approximately one parasite/L) were processed. Samples collected with an automated water sampler (ISCO) were treated as technical replicates and only one sample of the three taken at each site for each time point was sequenced. Samples taken in 2006 and 2007 were grab samples; these were treated as biological replicates and therefore all three replicate samples were sequenced. Samples that did not yield a Cq value in the initial run were diluted 1:10 (if the 1-10 dilution was likely to remain within our detection limits for the assay) and re-assayed.
At the Beaver Creek index site (Fig. 1.3.1), three of the four genotypes were detected. In all years (2006-2011) genotype I was dominant, followed by genotype II. In September 2008 and 2009, a relatively high amount of genotype II was detected. In 2007 (Fig. 1.3.2), genotypes I and II were detected at all five index sites; genotype III was detected at three of the five sites, most frequently (only at very low levels) at the lowermost index site, KTC; and genotype O was only detected in water samples collected at the uppermost index site, KKB. In 2009 (Fig. 1.3.3), genotype I and II were lower than in 2007, except at the lowermost site KTC, where levels were higher. Genotype III was detected sporadically at all five index sites, whereas genotype O was not detected at any.

**Fig. 1.3.1.** Density of *Ceratomyxa shasta* ITS1-genotypes in Klamath River water samples collected at the Beaver Creek index site from 2006 – 2011.
Fig. 1.3.2. Density of Ceratomyxa shasta ITS1-geno types in Klamath River water samples collected at five index sites in 2007. KKB=Klamathon Bridge, KBC=upstream of Beaver Creek, KSV=Seiad Valley, KOR=Orleans, KTC=Tully Creek.

Fig. 1.3.3. Density of Ceratomyxa shasta ITS1-geno types in Klamath River water samples collected at five index sites in 2009. KKB=Klamathon Bridge, KBC=upstream of Beaver Creek, KSV=Seiad Valley, KOR=Orleans, KTC=Tully Creek.
**Task 1.4 Polychaete abundance and infection prevalence**

Assays to determine prevalence of *C. shasta* infection have been completed for years 2006-2011. Infected *M. speciosa* were detected in all years, but not in all months or at all sites (Table 1.4.1). Overall POI (May-September) ranged from 0.04% (2011) to 2.8% (2009) and was < 1% in all years except 2008-2009. Infected *M. speciosa* were more commonly detected in June than May or September ($X_2=11.9$, $p<0.05$) and in TOH samples ($X_2=6.01$, $p<0.05$).

In general, POI in *M. speciosa* was consistent with sentinel fish exposure data that demonstrated disease risk to fish was high in 2008-2009 and lower in other years. POI in *M. speciosa* was >1% in 2008-2009 and <1% in other years but these differences were also not significant ($H_5=3.8$, $p=0.5$). The highest POI values were observed in 2009 (Table 1.4.1; 10% in May, followed by 6% in June), followed by 2008. However, because 2009 values were based on few polychaetes (e.g., 10 in May), we suggest POI values obtained for 2008 samples (~1%) are likely more representative of POI in *M. speciosa* during high fish loss years. Estimated densities of infected *M. speciosa* (calculated as density of *M. speciosa* * POI) were not consistent with high disease risk to fish in 2008-2009 (Figure 1.4.1). Estimated densities of infected *M. speciosa* were highest in 2007, followed by 2008, 2009-2010, and lowest in 2011.

We did not detect significant correlations between POI and density of *M. speciosa* ($r_s=0.05$, $p>0.05$) or median size of *M. speciosa* ($r_s=0.77$, $p>0.05$). However, POI was significantly correlated with peak flow ($r_s=-0.69$, $p=0.04$).
Table 1.4.1. Prevalence of *C. shasta* infection (POI, shown in boldface type) in *M. speciosa* from monitoring sites from 2006-2011 shown by site, year, and month. Total number of *C. shasta* positive molecular pools, number of molecular pools tested, and number of individual *M. speciosa* in each pool are also shown for each month and year. NS denotes sites where POI was not measured because sampling did not occur. NP denotes sites that were sampled but *M. speciosa* were not found.

<table>
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Figure 1.4.1. Estimated densities of *C. shasta*-infected *M. speciosa* for the Klamath River from a) 2007, b) 2008, c) 2009, d) 2010, and e) 2011 at I-5 bridge (upstream of confluence with Shasta), Tree of Heaven (downstream of confluence with Shasta), and Seiad Valley (downstream of confluence with Scott River).

**Task 1.5 Project coordination**

A conference call with collaborators from the Ca/Nv Fish Health Center and US Fish and Wildlife Service occurred in January. Data were shared, study plans for upcoming research, outstanding questions and the annual Klamath Fish Health Meeting were discussed.
Objective 2. Develop a model to address critical uncertainties in parasite transmission in relation to environmental parameters such as temperature and flow.

Task 2.1 Laboratory validation of field estimated transmission rate
The laboratory experiments have been completed and a manuscript has been submitted.

**Velocity challenge:** Iron Gate Hatchery Chinook salmon were exposed to the actinospore stage of *C. shasta* at four different velocities (0.05, 0.18, 0.32, and 0.43 m/sec). The goal of this study was to validate the transmission estimates observed from the previously conducted field exposures. The total number of parasites transmitted differed between velocities ($F_3 = 10.55, P = <0.0001$) and was higher at the slower velocities compared to the faster velocities (Fig. 2.1.1a.). Not only were fewer parasites transmitted at higher velocities, but the prevalence of infection was almost 3-fold lower at the higher velocities (27 and 20%) than at the slower velocities (75% at both lower velocities). The average transmission rates were greatest and approximately equal at 0.05 and 0.18 m/sec and decreased at the 2 higher velocities ($F_3 = 9.226, P < 0.0001$) (Fig. 2.1.1b.). As the doses were more consistent in the laboratory trials, we were able to directly observe an inverse relationship between velocity and transmission. We also identified a threshold between 0.18 and 0.32 m/sec where transmission efficiency was greatly reduced. As with the previous field experiments, an inverse relationship between water velocity and transmission rate was observed in this laboratory experiment. Due to the limited supply of infectious water we were unable to replicate the 3h exposure period conducted in the field setting and therefore our laboratory transmission estimates were not comparable to the field estimates. We also observed a velocity threshold as both the transmission rate and prevalence of infection dramatically decreased between 0.18 and 0.32 m/sec. This suggests that fish (i.e. larger or older) that spend more time in the thalweg of the channel, where flows are > 0.3 m/sec, may become less heavily infected than those that inhabit areas of slower velocities (e.g. proximal to banks, slow flowing pools, etc.).
Fig. 2.1.1. The number of Ceratomyxa shasta transmitted (a) and transmission rates (b) for Chinook salmon ( Oncorhynchus tshawytscha ) at four different velocities (0.05, 0.18, 0.32, and 0.43 m·sec$^{-1}$) in a laboratory challenge. The solid lines are the medians, the boxes cover the interquartile range, the bars the 5% to 95% ranges, and the dots are outside two standard errors.

**Temperature challenge:** Trout Lodge rainbow trout were exposed to the actinospore stage of *C. shasta* at 4 temperatures (11, 14, 18, and 22°C) to determine if there was a relationship between transmission rate and water temperature. Total number of parasites transmitted was low in the 11°C, intermediate in the 14 and 22°C and high in the 18°C treatments ($F_3 = 10.78, P < 0.0001$) (Fig. 2.1.2a.). As with total parasites transmitted, there was a difference in transmission rates among the temperature groups ($F_3 = 11.03, P < 0.0001$) (Fig. 2.1.2b.). The highest transmission rate occurred in the 18°C group and, interestingly, the 14 and 22°C groups had very similar mean transmission rates. Both parasites transmitted and transmission rate appear to be maximized around 18°C. We hypothesized that increasing water temperature would cause an increase in transmission as a function of increased ventilation of the fish at higher temperatures. Increased ventilation would cause more water to move over the gills, increasing the number of actinospores potentially contacting the gill surface. Although this hypothesis was not fully supported, we defined an optimal temperature for transmission (around 18°C).
Fig. 2.1.2. The number of *Ceratomyxa shasta* transmitted (a) and transmission rates (b) for rainbow trout (*Oncorhynchus mykiss*) at four different temperatures (11, 14, 18, and 22°C) in a laboratory challenge. The solid lines are the medians, the boxes cover the inter-quartile range, the bars the 5% to 95% ranges, and the dots are outside two standard errors.

The above forms part of the manuscript ‘Estimation of Transmission Dynamics of the *Ceratomyxa shasta* Actinospore Stage to the Salmonid Host’, by R. Adam Ray and Jerri L. Bartholomew, that has been accepted for publication in *Parasitology*.

**Task 2.2 Model completion and sensitivity analysis**

A manuscript is in preparation.

**Objective 3. Develop a qPCR assay to differentiate between the different ITS1 strains of *C. shasta*.

**Task 3.2 Develop a strain-specific molecular assay for *C. shasta***

Polychaete mesocosms were established to create strain-specific parasite cultures. These were successfully utilized to infect fish in laboratory experiments.

We have developed a SYTO9 qPCR assay which improved our genotyping capabilities. The regular SSU-qPCR quantifies total *C. shasta* present in a water sample. We used a separate novel ITS1-qPCR followed by direct sequencing of the amplicons to determine the
proportion of ITS1 genotypes present. We developed an intercalating-dye-based qPCR rather than a probe-based qPCR to capture all possible genotypes, not just known ones. We used SYTO9 in lieu of the standard SYBR Green dye because the latter has demonstrated dye concentration and DNA concentration issues (Monis et al. 2005).

The rRNA gene arrays of all known C. shasta ITS1 genotypes (0, I, II, III and respective subgenotypes) were aligned in BioEdit (Hall, 1999) and novel primers were designed manually that spanned the variable ITS1 region. Primer specificity was assessed in silico using BLAST (http://blast.ncbi.nlm.nih.gov/) and combinations of three forward and three reverse primers were assessed in vitro. The chosen pair, forward primer CsgenF4 5’ GGCAGAATTATTTGTCG 3’ and reverse primer CsgenR1 5’AGGGATCCACCGTAAAC 3’ amplified 161 bp. We assessed primer performance at a range of annealing temperatures (56, 58, 62°C) and times (45 sec, 1:15min, 1:30min). The optimized reaction and cycle conditions follow. The master mix for a single 20µL ITS1-qPCR was: 10µL of stock TaqMan® Gene Expression Master Mix (ABI, Life Technologies), 4.7µL of molecular grade water, 1.6µL of 10µM forward and reverse primer, 0.5µL of 10mg/mL BSA and 0.6µL of 1.5µM of SYTO9 green fluorescent nucleic acid stain (Invitrogen, Life Technologies, Grand Island, NY) and 1µL of extracted DNA sample.

Reactions were run in ABI Prism® 96-well optical reaction plates (Foster City, CA) on an ABI 7300 Real Time PCR System. The cycling conditions were: 2 min hold at 50°C, 10 min hold at 95°C followed by 40 cycles of 95°C for 15 sec and 58°C for 1 min 30 sec. A dissociation step was added to allow for visualization of the product. This consisted of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. A FAM reporter was selected as per Monis et al. (2005), no quencher and ROX was used as the passive reference. Samples with dissociation curves between 72-73°C were diluted 1:20 with molecular grade water and submitted for Sanger sequencing with CsgenF4 at the Center for Genomics and Bioinformatics at Oregon State University, Corvallis, OR using a BigDye® Terminator v.3.1. Cycle Sequencing Kit with an ABI Prism® 3730 Genetic Analyzer, ABI Prism® 3730 Data Collection Software v.3.0 and ABI Prism® DNA Sequencing Analysis Software v.5.2. Parasite genotypes were differentiated and proportions were assessed according to Atkinson and Bartholomew (2010a). Data generated using this new assay are presented in Task 1.3.

Objective 4. Provide finer resolution of the infectious zone in the lower Klamath River

Task 4.2 Synthesis of multi-year water sample data

Combined water sample and sentinel fish exposure data from 2006-2010 were examined for relationships between parasite density and infection severity infection in Chinook, coho and susceptible rainbow trout. These analyses have been published in Applied and Environmental Microbiology: Hallett, S.L., Ray, R.A., Hurst, C.N., Holt, R.A., Buckles, G.R., Atkinson, S.D. & Bartholomew, J.L. (2012) Density of the Waterborne Parasite, Ceratomyxa shasta, and its Biological Effects on Salmon 78: 3724-3731. Further analyses are being coordinated with current modeling efforts in the basin (e.g. SALMOD).
PUBLICATIONS, 2012 or in press

Ray, A.R and J. L. Bartholomew. 2013. Estimation of transmission dynamics of the Ceratomyxa shasta actinospore to the salmonid host. Parasitology. Accepted


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