

FY2005 Investigational Report:
Longevity of *Ceratomyxa shasta* and *Parvicapsula minibicornis* actinospore infectivity in
the Klamath River: April – June 2005.



J. Scott Foott*, R. Stone, E. Wiseman, K. True and K. Nichols
U.S. Fish & Wildlife Service
California – Nevada Fish Health Center
24411 Coleman Hatchery Road
Anderson, CA 96007
(530) 365 – 4271 , Scott_Foott@fws.gov
Fax 530 – 365 – 7150

February 2006

* Direct Correspondence

Summary: Infectious *Ceratomyxa shasta* and *Parvicapsula minibicornis* actinospores were present in Klamath River samples collected in April, May, and June 2005. Juvenile Chinook salmon, exposed to river water maintained at ambient Klamath River temperature for 0,4,24, 72, and 168 h (7 days), developed asymptomatic infections from both parasites. Elevated water temperature (18°C) in June may have caused the observed reduction in actinospore viability as both *C. shasta* and *P. minibicornis* infection markedly declined in fish exposed to water held for over 72 h. As judged by the incidence of infection for both parasites, the number of infectious actinospores tended to increase or remain steady over the spring. *Ceratomyxa shasta* infections were characterized by the presence of very few trophozoites within granulomatous foci of the visceral adipose tissue and were consistently observed outside of the intestine. Similarly, low numbers of *P. minibicornis* were observed in the kidney but were not associated with inflammation. *Parvicapsula minibicornis* DNA was consistently detected by QPCR in filtered water samples collected each month and from each time post – transfer. This data and the high incidence of infection observed in the exposed fish indicate that *P. minibicornis* actinospores were at a relatively high concentration in the river during the spring. In contrast, *C. shasta* DNA was only detected in half of the water sample sets and its detection did not correspond well to *C.shasta* infectivity. An approximately 3x increase in river flow from the April to the May water collection was not associated with reduced actinospore detection (particularly *P. minibicornis*) or incidence of infection for both parasites. Actinospores of these myxosporean parasites have the potential to infect salmonids for at least 7 days after release from the alternate polychaete host.

The correct citation for this report is:

Foott JS, R. Stone, E Wiseman, K. True and K. Nichols. 2006. FY2005 Investigational report: Longevity of *Ceratomyxa shasta* and *Parvicapsula minibicornis* actinospore infectivity in the Klamath River: April – June 2005. U.S. Fish & Wildlife Service California – Nevada Fish Health Center, Anderson, CA.

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Introduction:

Juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in the Klamath River incur a high incidence of infection from 2 myxozoan parasites, *Ceratomyxa shasta* and *Parvicapsula minibicornis* (Nichols & Foott 2005). These salmonid parasites occur in a number of Pacific Northwest watersheds and their lifecycles include the polychaete, *Manayunkia speciosa*, as an alternate host (Hoffmaster et al. 1988, Bartholomew et al. 1997, Jones et al. 2004, Bartholomew et al. *in press*). The actinospore, a stage that is infectious to salmon, is released from infected polychaetes into the water column and infections by *C.shasta* can occur from spring through fall at water temperatures $\geq 7^{\circ}\text{C}$ (Ching & Munday 1984, Hendrickson et al. 1989). Seasonal infectivity data of *P. minibicornis* has not been reported but appears to be similar to *C. shasta* in the Klamath River.

Hendrickson et al. (1989) describe *C. shasta* distribution in California to include the San Joaquin, Sacramento, Pit, and Klamath River systems. In the Klamath basin, *C. shasta* infection has been detected in juvenile salmonids from the mouth of the Klamath River to Iron Gate dam (rkm 190), Copco reservoir, both Klamath and Agency Lakes, and the lower reaches of the Williamson and Sprague Rivers (Hendrickson et al. 1989, Bartholomew et al. 2003). No infected juvenile Chinook smolts have been detected in health monitoring work in the Scott, Shasta, and Trinity Rivers (Foott et al. 2001, Nichols et al. 2003). High mortality due to ceratomyxosis has been observed in juvenile Chinook salmon out-migrants in the Klamath R. basin (Foott et al. 1999 and 2002, Nichols et al. 2003). In 2004, a pilot study to examine the diurnal infectivity pattern of *C. shasta* and *P. minibicornis* actinospores in the Klamath River demonstrated no diurnal changes (Appendix 1). Groups of salmon were sequentially exposed for 6 h in the Klamath River over a 24h period and compared to fish held in the river for the full 24h. All groups experienced clinical infections of both *C. shasta* and *P. minibicornis*. The results of this study prompted us to change the objectives of our proposal from examining diurnal patterns of infectivity to actinospore longevity (Appendix 2).

We addressed 3 questions: 1) Does the relative infectivity of *P minibicornis* and *C. shasta* in the Klamath River change during the spring (as water temperature increase), 2) Is there a correlation between the presence and relative concentration of parasite DNA in the exposure water and fish infection, and 3) How do Iron Gate Chinook juveniles respond to limited parasite exposures?

Methods:

Exposure conditions –Juvenile Chinook salmon, Klamath River stock, were obtained from Iron Gate Hatchery in April 2005. Mean fork length of Chinook used in the 3 challenges ranged from 65 to 82 mm. On 18 April, 16 May, and 15 June 2005, 770 L of river water was pumped into a tank mounted in a truck bed. Water was collected at Fisher’s resort, approximately 3 km upstream of Beaver creek at rkm 262. Water was aerated during the 2 h transport to the CA-NV Fish Health Center wet lab and later held at ambient river temperature within a chiller equipped water bath. Temperature, in both the river water tank and exposure aquaria, was monitored every hour with Onset™ Stowaway temperature loggers. Each exposure group of 12 -15 fish

was held at a ratio of 1 fish / L river water for 24 h and then reared for 18 to 25d in temperature controlled flow-through aquaria supplied with ozone-treated water. Water within the storage tank was mixed prior to the removal of the exposure volumes and 1 L samples to assess the abundance of parasite DNA. Replicate groups were exposed to river water at 0, 4, 24, 72, and 168 h after river water collection. The zero hour exposure was performed by transporting fish to the pump site and placing them in ice chests containing aerated river water. In order to reduce the occurrence of columnaris disease, each exposure group received a 10 min prophylactic bath of 1 ppm furanase one day following the exposure period. Fish were fed a commercial salmon diet at 1% body weight per day.. Mortalities were frozen for later analysis of parasite DNA by Quantitative Polymerase Chain Reaction (PCR).

Necropsy – After 18 – 25 days post-exposure (dpe), salmon were euthanized by an overdose of MS222, examined for pale gill (anemia) and internal abnormalities such as intestinal hemorrhage or swollen kidney, and the intestinal tract and posterior kidney was fixed for histological examination. Prior to the fixation step, a 2 mm section of lower intestine was removed by DNA-free tools (washed in DNA-AWAY[™] {Molecular BioProducts, San Diego, CA} and rinsed twice in distilled water) for storage at -70°C as a PCR archive sample. A subset of the intestinal samples were tested for *C. shasta* 18S rDNA by the Polymerase Chain Reaction (PCR) method of Hallett and Bartholomew (in press). DNA was extracted with an Applied Biosystem Model 6100 Nucleic Acid Prep Station using reagents and procedures provided by Applied Biosystem. Intestinal tract (including the caeca) and kidney was 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).

Parasite DNA detection in Water samples – Immediately after removing water for the 4 to 168 h exposures, two 1-L samples were collected from the river water storage tank. Each liter was filtered through a 47mm 5.0 µm nitrocellulose filter (Millipore Inc.) and the filter stored at -70 C until DNA extraction. During May, the high suspended solid content required that some liter samples be divided into half or less for filtration.

Quantitative Polymerase Chain Reaction assays - Quantitative Polymerase Chain Reaction (QPCR) utilizes a unique fluorogenic probe with a reporter molecule and quencher dye, commonly called the TaqMan® probe. Probes and primers were developed against 18S ribosomal DNA sequences of *C. shasta* (Hallett in press) and *P. minibicornis* (Kimberly True, unpublished data) based on previously published PCR primer sequences utilized in standard PCR assays (Palenzuela et al. 1999, St.Hiliare et al. 2002). These specific primers and probe sets are used to detect and quantify parasite DNA in real-time using a sophisticated fluorometer and specialized software (Applied Biosystem, Foster City, CA). In the QPCR assay, samples containing target DNA undergo amplification, increasing the quantity of specific DNA and associated fluorescence during each reaction cycle. Fluorescent Unit change (? Rn) is measured at each cycle and positive test results are reported as Cycle Threshold (Ct), the point

when fluorescent signal surpasses normalized background levels. The 25 μ L reaction mixture consisted of: 12.5 μ L Taqman universal master mix (Applied Biosystems, Foster City, CA), 800nM of each primer, 200nM Taqman® Probe, 3 μ L molecular grade water and 5 μ L DNA template (approximately 300ng / reaction). Tests were run in an Applied Biosystems 7100 Sequence Detection System®. Detection thresholds are set at R_n of 1000 fluorescent units (FU) which occur at (Ct) values of 38-39.

Results:

C. shasta infection - Trophozoites were detected in salmon (both by histology and PCR) from each exposure at $\geq 39\%$ prevalence of infection (Fig. 1). One exception was the June 168h exposure group with only a 7% prevalence of infection. There was considerable variation in the prevalence of infection observed in the replicates with 7 of 15 replicates having differences of more than 2 infected fish (Table 1). The zero and 4h exposure groups showed similar infectivity trends for the 3 months (June < April < May) with the 4h groups consistently having the highest prevalence of infection (Fig. 1). The 24 and 72 h groups also demonstrated similar monthly infectivity trends (April < May < June) that were different from the 0 and 4h exposures. The June 168h exposure group had markedly lower prevalence of infection (7%) than either April (39%) or May (50%).

Low numbers of trophozoites were consistently observed within inflammatory foci of visceral adipose tissue or on the intestinal serosa in those salmon diagnosed with *C. shasta* (Fig 2.). Trophozoites, within the intestine, were observed in 2 of the 422 sections read in the study. Focal inflammation outside of the intestinal tract without parasites was also commonly observed in the intestine sections and PCR assay of the lower intestine (rectum) of these fish were typically positive. In the June exposure experiment, a total of 17 mortalities occurred after 2 weeks of rearing. *C. shasta* DNA was detected in the majority of the 24 and 72h mortalities (PCR+: 0h= 0 of 1, 4h= 0 of 1, 24h= 5 of 6, 72h= 7 of 9).

Between 50 – 75% of each sample group was assayed by both QPCR of the lower intestine and by microscopic examination of the remaining intestinal tract. PCR detected an additional 13 – 71% positive fish (Table 2). The low numbers of *C. shasta* trophozoites observed in sections suggest that many light infections were not observed by histology. Matching results occurred in 21 – 87% of the sample groups while false negative results (PCR -, Histology +) were relatively infrequent (0 – 17%).

There was an 8 °C difference in river temperature between the April and June collection (Table 3). River temperature at the time of collection was 10.8°C on 18April, 14.1°C on 16May, and 18.3°C on 15June. We were able to maintain the river water within a daily mean of 1.5°C of the collection temperature. In response to rain events, there was a marked increase in Klamath River flows during May. On the day of water collection, flows (cfs) measured at Iron Gate Dam were 1520 on 18April, 4420 on 16May, and 1210 on 15June (<http://waterdata.usgs.gov/ca/nwis>). Despite the high flows of May, *C. shasta* infectivity was higher than that of April for all storage times.

Figure 1. Prevalence of *C.shasta* infection in salmon exposed to Klamath River water aged for 0, 4, 24, 72 and 168 h. Water was collected on 18April, 16 May, and 15June 2005. Parasite infection was determined by microscopic examination of intestinal tract sections and from a sub-set of lower intestine samples assayed by polymerase chain reaction.

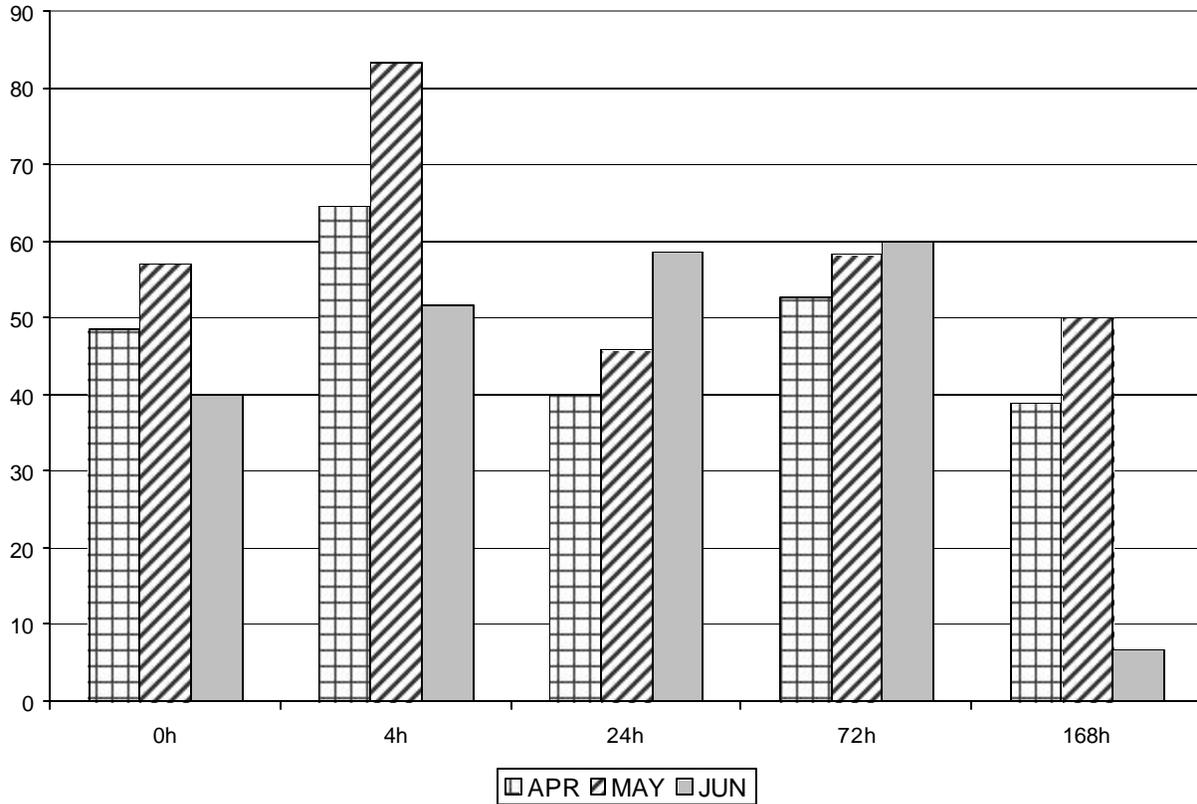


Table 1.

Prevalence of *C. shasta* infection in replicate groups (A and B) of salmon exposed to Klamath River water aged for 0, 4, 24, 72 and 168 h. Water was collected on 18April, 16 May, and 15June 2005. Parasite infection was determined by microscopic examination of intestinal tract sections (HISTO) and from a sub-set of lower intestine samples assayed by polymerase chain reaction (PCR). PCR data represents the additional positive samples (negative by histology and positive by PCR) attributed to an exposure group (PCR+ additions). Data recorded as number positive / total sample size (%).

	HISTO (A)	HISTO (B)	HISTO combined	PCR+ additions
18April				
0h	2 / 17 (11)	8 / 18 (44)	10 / 35 (29)	7 / 18 (39)
4h	5 / 15 (33)	4 / 16 (25)	9 / 31 (29)	11 / 18 (61)
24h	4 / 18 (22)	3 / 17 (18)	7 / 35 (20)	7 / 18 (39)
72h	4 / 18 (22)	7 / 18 (39)	11 / 35 (31)	8 / 18 (44)
168h	2 / 18 (11)	0 / 18 (0)	2 / 36 (6)	12 / 18 (67)
16May				
0h	2 / 16 (13)	2 / 12 (17)	4 / 28 (14)	12 / 17 (71)
4h	1 / 6 (17)	2 / 12 (17)	3 / 18 (17)	12 / 18 (67)
24h	0 / 12 (0)	1 / 12 (8)	1 / 24 (4)	10 / 17 (59)
72h	4 / 12 (33)	2 / 12 (17)	6 / 25 (25)	8 / 18 (44)
168h	3 / 12 (25)	0 / 12 (0)	3 / 24 (13)	9 / 18 (50)
15June				
0h	1 / 16 (6)	3 / 14 (21)	4 / 30 (13)	8 / 14 (57)
4h	1 / 14 (6)	3 / 14 (21)	5 / 28 (18)	10 / 14 (71)
24h	3 / 8 (38)	1 / 15 (7)	4 / 23 (17)	8 / 14 (57)
72h	1 / 11 (9)	1 / 10 (10)	2 / 21 (10)	9 / 15 (60)
168h	0 / 15 (0)	0 / 14 (0)	0 / 29 (0)	2 / 15 (13)

Figure 2. Granuloma containing *Ceratomyxa shasta* trophozoites (arrows) within visceral adipose tissue of salmon from April 4 h exposure, (bar = 20 μ m, H&E stain).

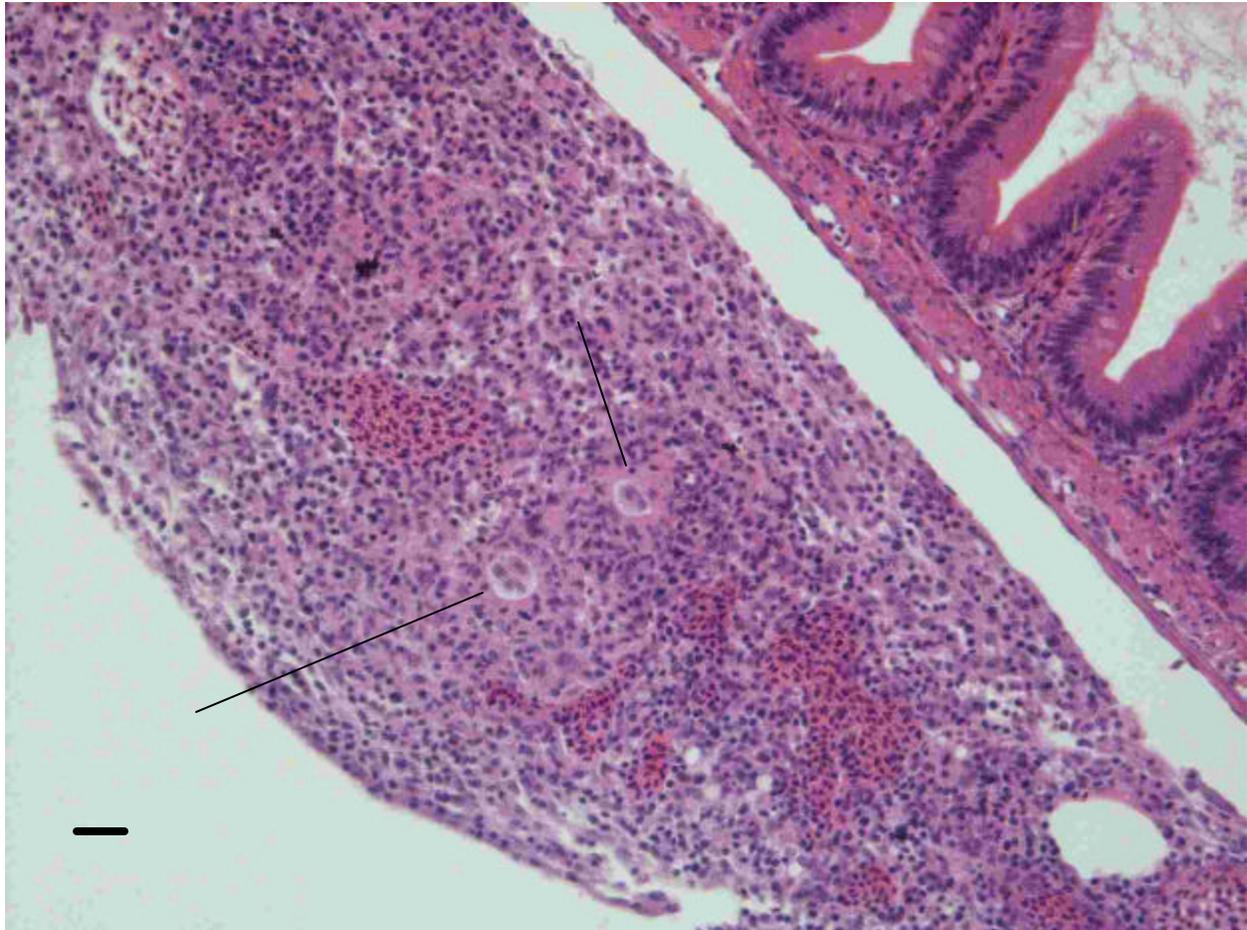


Table 2.

Relationship between QPCR assay results for *C. shasta* and histological detection of the parasite. A portion of the lower intestine was tested by QPCR and compared with microscopic examination of the remaining intestinal tract. Data reported as number of QPCR samples (P+ or P-) meeting criteria of histology negative (H-) or positive (H+) and percent of QPCR sample set (%).

	PCR samples	PCR additions H - / P + (%)	PCR match H+/P+ or H-/P- (%)	PCR false negative H+/P- (%)
<u>April</u>				
0h	18	7 (39)	11 (61)	0
4h	18	11 (61)	7 (39)	0
24h	18	7 (39)	11 (61)	0
72h	18	8 (44)	8 (44)	2 (11)
168h	18	12 (67)	4 (22)	2 (11)
<u>May</u>				
0h	17	12 (71)	3 (18)	2 (12)
4h	18	12 (67)	5 (28)	1 (6)
24h	17	10 (59)	7 (41)	0
72h	18	8 (44)	8 (44)	2 (11)
168h	18	9 (50)	6 (33)	3 (17)
<u>June</u>				
0h	14	8 (57)	5 (36)	1 (7)
4h	14	10 (71)	3 (21)	1 (7)
24h	14	8 (59)	6 (43)	0
72h	15	9 (60)	5 (33)	1 (7)
168h	15	2 (13)	13 (87)	0

Table 3. Temperature (°C) of river water stored for 7 d and in aquaria during the 18 - 25 d rearing period for exposures groups in April, May, and June. Data reported as mean (Std.dev.).

	River Water	Aquaria
April	10.6 (0.4)	14.4 (1.4)
May	15.3 (1.0)	17.8 (1.5)
June	16.8 (1.4)	18.1 (1.0)

P. minibicornis infection - This myxosporean parasite was observed in $\geq 96\%$ of the 0 – 72h fish kidney sections of all exposures (Table 4). At 168h, the prevalence dropped considerably to 28% in April, 34% in May, and 0% in June. It is likely that some of the June 168h fish were lightly infected. No PCR analysis was performed on these samples. Parasite numbers tended to decrease as the river water was aged. Glomerulonephritis was only observed in 2 fish in the June 0 h exposure group. Neither *P. minibicornis* or *C. shasta* trophozoites were observed in sections from 12 non-exposed salmon sampled in April.

Correlation of water samples to infectivity- The detection of *C. shasta* DNA in water samples was variable (50% of sample sets) and did not correspond to fish infectivity (Table 5). DNA was only detected in one replicate from the June collection. The C_T value of positive samples ranged from 35.1 to 39.5 (Table 6). *Parvicapsula* DNA was detected in every sample set and from 21 of 24 duplicates. While the *P. minibicornis* infection declined sharply at 7d, DNA was detected in the corresponding water samples. The C_T value of positive samples ranged from 33.6 to 38.6 (Table 6).

Table 4.

Prevalence of *P. minibicornis* infection in replicate groups (A and B) of salmon exposed to river water aged for 0, 4, 24, 72 and 168 h. Water was collected on 18April, 16 May, and 15June 2005. Parasite diagnosis was by microscopic examination of kidney sections (HISTO). Data recorded as number positive / total sample size (%).

	HISTO (A)	HISTO (B)	HISTO combined
18April			
0h	17 / 17	18 / 18	35 / 35 (100)
4h	15 / 15	16 / 16	31 / 31 (100)
24h	18 / 18	17 / 17	35 / 35 (100)
72h	17 / 18	18 / 18	35 / 36 (97)
168h	5 / 18	5 / 18	10 / 36 (28)
16May			
0h	16 / 16	12 / 12	28 / 28 (100)
4h	6 / 6	12 / 12	18 / 18 (100)
24h	12 / 12	12 / 12	24 / 24 (100)
72h	12 / 12	11 / 12	23 / 24 (96)
168h	6 / 12	2 / 12	8 / 24 (33)
15June			
0h	16 / 16	14 / 14	30 / 30 (100)
4h	14 / 14	14 / 14	28 / 28 (100)
24h	8 / 8	15 / 15	23 / 23 (100)
72h	11 / 11	10 / 10	21 / 21 (100)
168h	0 / 15	0 / 14	0 / 29 (0)

Table 5.
 QPCR results for *C. shasta* and *P. minibicornis* DNA in 1L replicate (A and B) river water samples collected on 18April, 16 May, and 15June 2005 and aged for 4, 24, 72 and 168 h. CT values > 40 recorded as undetected (UND).

	<i>C. shasta</i>		<i>P. minibicornis</i>	
	(A)	(B)	(A)	(B)
18April 4h	UND	UND	UND	+
24h	+	+	+	+
72h	+	+	+	+
168h	UND	UND	+	+
16May 4h	+	+	+	+
24h	+	UND	+	+
72h	UND	UND	+	+
168h	+	+	+	+
15June 4h	UND	UND	+	+
24h	+	UND	+	UND
72h	UND	UND	+	+
168h	UND	UND	+	UND

Table 6.

Cycle threshold (Ct) values for *C. shasta* and *P. minibicornis* DNA in 1L replicate (A and B) river water samples collected on 18April, 16 May, and 15June 2005 and aged for 4, 24, 72 and 168 h. CT values > 40 recorded as undetected (UND). Volume of filtrate is listed for sample < 1 L.

	<i>C.shasta</i>				<i>P.minibicornis</i>			
	(A)		(B)		(A)		(B)	
18April								
4h	UND		UND		UND		34.8	
24h	35.1		39.5		34.9		34.5	
72h	39.2		36.9		35.2		35.2	
168h	UND		UND		37.2		36.5	
16May	700mL	300 mL	500mL	500 mL	700mL	300 mL	500mL	500 mL
4h	UND		36.2		36.4		38.6	
24h	<u>500mL</u>		<u>500 mL</u>		<u>500mL</u>		<u>500 mL</u>	
	39.2		UND		36.6		38.2	
72h	<u>500mL</u>	<u>500mL</u>	<u>500mL</u>	<u>500mL</u>	<u>500mL</u>	<u>500mL</u>	<u>500mL</u>	<u>500mL</u>
	UND		UND		UND		UND	
	36.7		35.0		33.8		35.5	
168h	<u>1000mL</u>		<u>1000mL</u>		<u>1000mL</u>		<u>1000mL</u>	
	36.9		38.3		37.6		38.9	
15June								
4h	UND		UND		37.1		38.3	
24h	36.3		UND		33.6		UND	
72h	UND		UND		36.2		35.3	
168h	UND		UND		37.4		UND	

Discussion:

Markiw and Wolf (1983) were the first researchers to describe the 2 phase lifecycle of myxosporeans: actinosporean stage within an invertebrate (alternate) host and myxosporean stage in the teleost host. Since this paper, numerous reports have been published describing the 2 host life cycle of other myxosporeans that involve such invertebrates as oligochaetes, polychaetes, and bryozoans (Whitaker et al. 2005, Bartholomew et al. 1997, Canning et al. 2000). In this 2 host life cycle, actinospores are released from the invertebrate host, infect the fish host and later develop into myxospores that are infective to the invertebrate. Thus both actinospore and myxospore can be in the water column. *Manayunkia speciosa* is the freshwater polychaete (alternate) host for *C. shasta* and *P. minibicornis* (Bartholomew et al. 1997 Bartholomew et al. 2006). These small tube building polychaetes are filter feeders and have been found throughout the Klamath River system (R. Stocking, Oregon State University, personal communication).

Infectious *C. shasta* and *P. minibicornis* actinospores were present in the Klamath River samples collected in April, May, and June 2005. As judged by the incidence of infection for both parasites, the number of infectious actinospores tended to increase or remain steady over the spring. An approximately 3x increase in river flow from the April to the May collection did not result in a decline of either actinospore detection (particularly *P. minibicornis*) or incidence of infection for both parasites. This data would suggest that increased spring flows may not be effective in reducing parasite infection rates by themselves however higher flow could act to speed out-migration and thereby reduce the duration of exposure for smolts.

The most obvious seasonal trend observed in this study was the markedly reduced longevity of both *C. shasta* and *P. minibicornis* actinospores in the June 168h exposure group. The warmer water temperatures (18°C) in June may have reduced the duration of actinospore viability. Ratliff (1983) reported that *C. shasta* infectivity of Deschutes river water lasted up to 7 d after transfer from the river when mean water temperature was 11°C (May) but was limited to 3 d post transfer in trout exposed to warmer (18°C) river water. Both *C. shasta* and *P. minibicornis* actinospores appear to be much more robust than the actinospore of *Tetracapsuloides bryosalmonae* (agent for Proliferative Kidney Disease of salmonids). This myxosporean is reported to be infectious for only 12 h in 14.5 – 17° C water (de Kinkelin et al. 2002).

The asymptomatic infections that occurred from the limited challenges (small volume of river water) conducted in our study demonstrate the resistant characteristic of the Klamath stock of salmon. There are numerous reports of resistance to ceratomyxosis by endemic stocks of salmonids (Bartholomew 1998, Buchanan et al. 1983, Ibarra et al. 1992, Zinn et al. 1977). High infectious load (long exposure duration and / or high actinospore concentration) has been shown to overwhelm such resistance and is a likely mechanism behind the high incidence of severe ceratomyxosis observed in Klamath River Chinook salmon juveniles (Ratliff 1981, Zinn et al. 1977). Unlike the

study fish, juvenile salmon in the river are exposed continuously to actinospores for the many weeks that they either rear or out-migrate in the Klamath River.

The few *C.shasta* trophozoites observed in the exposure fish sections were surrounded by granulomatous tissue indicating a successful host defense. The apparent low number of actinospores in the exposure volume was sufficient to infect the fish but did not result in disease. A similarly strong host defense was observed in a 2002 study in which salmon were injected with *C. shasta* trophozoites and later developed a inflammatory response that contained the parasite (Foott et al. 2004). In contrast to most specimens collected from the Klamath River, the study fish rarely had trophozoites within the intestinal tract. Rather the parasite was consistently found outside of the intestine and associated with adipose tissue or on the serosal surface of the lower intestine. The initial route(s) for invasion of the fish host has not been thoroughly documented. However, the presence of trophozoites on or within the intestinal epithelium in salmon collected early in the season or soon after release from Iron Gate hatchery suggests that ingestion of the actinospore is a likely avenue of invasion. Liyanage et al. (2003) reported that actinospores of *Thelohanellus hovorkai* would invade carp vascular system after oral intubation indicating the initial invasion site was the GI tract. The authors speculate that ingestion of infected oligochaetes would be a significant pathway for infection in the fish host. A similar mechanism may occur between *M. speciosa* and juvenile salmonids in the Klamath R. If the intestinal route is the primary invasion path, our observations suggest that the parasite migrates into the peritoneum within 18 to 25d of the initial infection.

Parvicapsula minibicornis DNA was consistently detected in filtered water samples collected each month and from each time post –transfer. This data and the high incidence of infection observed in the exposed fish indicate that *P. minibicornis* actinospores were at a relatively high concentration in the river during the spring. Interestingly the 168h exposure group *P minibicornis* infection rate dropped $\geq 67\%$ when compared to the early time periods, yet there was no significant decrease in the amount of DNA detected in the 168h water samples. This data points out the inherent limitation when attempting to estimate infectious load of the river from filtered water samples as this method cannot account for actinospore viability.

In contrast, *C. shasta* DNA was only detected in half of the water sample sets and appeared to have a non-homogenous distribution. An example of this uneven distribution occurred in one May 4h water sample that was divided into a 300 and 700 mL aliquot for filtration. The smaller aliquot tested DNA positive while no PCR detection of *C. shasta* DNA occurred in the larger volume. The filtration / QPCR assay of 1liter samples may lack the sensitivity to detect low numbers of actinospores that are sufficient for sub-clinical infections. Substances in the water (e.g. humic acid) could also inhibit the PCR assay (S. Hallett, Oregon State University personal communication). Non-homogenous distribution in the water sample could also help explain the different incidence of *C.shasta* infection among the replicate exposure groups. It is likely that a relatively low number (1?) of actinospores can produce an infection in the salmon. Ratliff (1983) performed a host density study to estimate the

concentration of infectious stage per unit of river water. Similar numbers of fish became infected when exposed to the same volume of river water regardless of the number of trout per replicate. He estimated that the Deschutes River had between 28 to 148 “infectious units”/m³ during the period of April through June 1979 with no apparent relationship to either flow or water temperature. The author also concluded that the mean time to death of exposed rainbow better correlated to the infectious load of the river than water temperature.

A direct method to determine actinospores concentrations in the river will be invaluable in answering questions about seasonality of the disease, habitat and environmental preferences of the alternative polychaete host, and challenge levels that lead to disease. The filtration / QPCR method was unable to consistently predict the *C.shasta* infectivity of the river water but performed well for *Parvicapsula* detection. This method does have potential to estimate the infectious potential of the river but needs further refinement. Increased volumes and composite sample strategies as well as better concentration or extraction methods are areas to investigate in the future. One aspect of this approach that cannot be controlled is the inability to distinguish between myxospore and actinospore in a water sample. Non- viable actinospores also appear to be detected by this method which would reduce the confidence in projecting infectivity potential from DNA concentration results.

Both *C. shasta* and *P. minibicornis* actinospores remain viable for a period long enough to be distributed for miles downstream from the shedding polychaete. Given the wide distribution of *M. speciosa* in the Klamath River, the range of infective waters is extensive in the main stem Klamath River.

Acknowledgements:

Partial funding for this work came from the Klamath River Basin Conservation Area Restoration Program (\$9000, project 2005- FP-016 FWS account 11230 – 1331-0000). We thank California Department of Fish and Game Iron Gate Hatchery for Chinook salmon and Paul Zedonis (USFWS) for his review and comments of this report.

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Appendix 1 Pilot experiment
 June 17 to 18, 2004 6 hr exposures 3 km above Beaver creek

Four sequential 6 hr exposures and a continuous 24hr exposure were conducted with 5 cages of 25 juvenile Chinook (IGH) at Fisher's resort (rkm 262) beginning at 12:35 on 17June. Prior to and following exposure fish were held within an aerated tank. Upon arrival at the wet lab on 18 June, the surviving fish were given a nitrofurazone bath to control *Columnaris* and reared for 18d at 18- 20°C. *Ceratomyxa shasta* infection was determined by microscopic examination of either a Giemsa stained imprint or histological section of the lower intestine and kidney.

Fish numbers / exposure time

exposure time	survivor	21June % mortality	Initial sample	6July Cs signs	Clinical Cs detection
6/17 12:35 -18:35	12	52%	11	9	4 of 4 tested
6/17 18:35- 24:20	11	56%	9	9	1 of 2 tested
6/18 24:20 -06:35	5	80%	5	2	2 of 3 tested
6/18 06:35 - 12:20	9	64%	3	3	2 of 2 tested
6/17 12:35 – 6/18 12:20	18	28%	10	9	2 of 2 tested
Unexposed controls	8	66%	7	0	0 of 4 tested

Results:

1. Holding fish in the tank prior to the river exposure appeared to be stressful and early mortality may be related to rapid temperature changes (IGH 13°C, River 21°C, tank of IGH water ranged from 13 – 15C).
2. A 6 hr exposure resulted in clinical ceratomyxosis and Parvicapsula infection regardless of diurnal period of exposure.
3. The continuous 24 hr group went to a clinical state sooner than the 6 hr groups (14 dpe) as would be expected in fish exposed to a larger infectious load.
4. No *Ceratomyxa shasta* trophozoites were observed in sections from three 18dpe survivors and one 6 hr exposure survivor had only a few trophozoites surrounded by an extensive granuloma (indicative of successful host defense).
5. Average Hematocrit of a 14dpe sample group (2 from each exposure) was 24.7% which is indicative of anemia.
6. Glomerulonephritis, due to Parvicapsula infection, was observed in 2 fish in the 24hr group.

Original proposal submitted in April 2004

PROJECT TITLE: Diurnal and seasonal abundance of the infectious stage of *Ceratomyxa shasta* in the mid-Klamath River.

OBJECTIVE: Determine the concentration of infectious actinospores in river water over several 24 hour periods by direct spore counts, parasite DNA concentration, and relative infectivity of exposed sentinel fish. The effect of water temperature and other seasonal conditions will be compared through multiple experiments in May and June. Prior to changes in flow management for disease moderation, a robust technique to characterize river infectivity and an understanding of the relationship between spore concentration and infectivity is necessary.

Modification of project - 2005

PROJECT TITLE: Longevity of infectivity for the infectious stage of *Ceratomyxa shasta* in the mid-Klamath River, seasonal and water temperature effects.

Modification of 2005-FP-16 “Diurnal and seasonal abundance of the infectious stage of *Ceratomyxa shasta* in the mid-Klamath River.”. In June of 2004, a pilot experiment demonstrated no diurnal differences in actinospore infectivity in the mid-Klamath. Groups of juvenile Iron Gate Hatchery Chinook were exposed to the Klamath River, 1 mile above Beaver Creek, every 6 hrs within a 24 hr period. One group was exposed for the full 24 hrs. All salmon contracted ceratomyxosis and infections by the kidney myxosporean *Parvicapsula minibicornis*. The 6 hr exposure was sufficient to induce lethal infections. We feel that the question of diurnal fluctuation posed in the original proposal has been answered and plan to direct our research at how long a given actinospore is infectious in the water column. Ratliff (1983) reported infection longevity of *C. shasta* in Deschutes River to range from 3 – 7 d depending on water temperature. This information would provide for risk modeling if infected polychaetes (alternate host) are concentrated in specific sites within the Klamath River **(i.e. if actinospores are released at mile X how many miles downstream are at risk).**

OBJECTIVE: Determine the concentration and infectivity of *C. shasta* actinospores in Klamath River water over a 1 week period in April, May, and June. The 3 sample times will allow for examination of seasonal and water temperature influences.