Journal of Aquatic Animal Health 28:75–84, 2016

ISSN: 0899-7659 print / 1548-8667 online
DOI: 10.1080/08997659.2015.1103803

ARTICLE

Production of *Ceratonova shasta* Myxospores from Salmon Carcasses: Carcass Removal Is Not a Viable Management Option

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Abstract

Severe infection by the endemic myxozoan parasite, *Ceratonova* (synonym, *Ceratomyxa*) *shasta*, has been associated with declines in and impaired recovery efforts of populations of fall-run Chinook Salmon *Oncorhynchus tshawytscha* in the Klamath River, California. The parasite has a complex life cycle involving a polychaete worm host as well as a salmon host. Myxospore transmission of this parasite, from salmon to polychaete, is a life cycle step during which there is a potential for applied disease management. A 3-year data set on prevalence, intensity, and spore characteristics of *C. shasta* myxospores was obtained from adult Chinook Salmon carcasses surveyed in the main stem of the Klamath River and three of its tributaries, Bogus Creek and the Shasta and Trinity rivers. Annual prevalence of myxospore detection in salmon intestines ranged from 22% to 52%, and spore concentration values per intestinal scraping ranged from 3.94×10^2 to 1.47×10^7 spores. A prevalence of 7.3% of all carcasses examined produced $>5.0 \times 10^5$ spores, and these carcasses with "high" spore counts accounted for 76–95% of the total spores in a given spawning season. Molecular analysis of visually negative carcasses showed that 45–87% of these samples had parasite DNA, indicating they contained either low spore numbers or presporogonic stages of the parasite. Myxospores were rarely found in carcasses of freshly spawned adults but were common in decomposed carcasses of both sexes. The date of collection or age (based indirectly on FL) did not influence detection. The longer prespawn residence time for spring-run Chinook Salmon compared with that for fall-run Chinook Salmon in the Trinity River was associated with higher spore loads. The dye exclusion method for assessing spore viability in fresh smears indicated an inverse relationship in spore integrity and initial spore concentration. A carcass-removal pilot project in Bogus Creek for 6 weeks in the fall of 2008 (907 carcasses removed) and 2009 (1,799 carcasses removed) failed to measurably influence the DNA quantity of *C. shasta* in targeted waters. Combined with the high numbers of carcasses that contributed myxospores, we therefore deemed that this labor-intensive approach is not a viable management option to reduce the infectivity of *C. shasta* in Chinook Salmon in the Klamath River.

The myxozoan parasite, *Ceratonova* (synonym, *Ceratomyxa*) *shasta* Noble 1950 (Atkinson et al. 2014), infects freshwater salmonid fishes and is enzootic to tributaries for anadromous fishes of the Pacific Northwest, including the Klamath River in California and Oregon (Hendrickson et al. 1989; Bartholomew 1998). Ceratomyxosis (enteronecrosis) is a disease that causes extensive mortality in juvenile Chinook Salmon *Oncorhynchus tshawytscha* in the Klamath River and could be a contributing factor to the decline of adult returns in the basin (Foott et al. 2004; Stocking et al. 2006; Fujiwara et al. 2011; Hallett et al. 2012). *Ceratonova shasta* has a complex life cycle, involving an invertebrate polychaete host

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Received January 23, 2015; accepted September 28, 2015

(*Manayunkia speciosa*) as well as the vertebrate salmon host (Bartholomew et al. 1997). Infected polychaetes release actinospores into the water where they attach to the salmon's gill epithelium, invade into the blood, replicate, and later migrate to the intestinal tract for further multiplication and sporogony (Bjork and Bartholomew 2010). Depending on actinospore genotypes and densities, innate host resistance, and water temperature, infected fish can develop varying degrees of enteritis and associated anemia (Udey et al. 1975; Bartholomew 1998; Foott et al. 2004; Bjork and Bartholomew 2009; Ray et al. 2010; Hallett et al. 2012). If the fish survives long enough (approximately 2 weeks at 18°C, 30 d at 12°C), sporogony occurs in the muscularis (primarily) with the production of myxospores (True et al. 2012). Myxospores released from the infected fish after death are ingested by the filter-feeding polychaete and complete their life cycle after invading the worm's gut epithelium (Meaders and Hendrickson 2009).

The Klamath River, between the confluence with the Shasta and Salmon rivers (Figure 1), has been identified to be highly infectious (hereafter referred to as "highly infectious zone": Stocking et al. 2006; Hallett et al. 2012) to salmon and is the focus for management actions to disrupt the parasite's life cycle. In August 2007, a multidisciplinary panel of fish disease experts and fishery managers developed a research plan that focused on management actions to reduce ceratomyxosis in natural Klamath River juvenile Chinook Salmon. One proposed management action was removal of adult salmon carcasses to reduce myxospore input into the system. The hypothesized effect of this action would be reduced polychaete infection and subsequent reduction of infectious actinospores and the associated mortality of juvenile fish the following spring. We examined the spatial and temporal distributions of myxospores in adult Chinook Salmon from the Klamath River basin to inform life cycle models (Ray et al. 2010) and optimize carcass removal efforts. We also examined potential myxospore production from adult Chinook Salmon and myxospore viability. Carcass removal occurred in Bogus Creek, a major salmon-spawning tributary of the Klamath River located upstream of the highly infectious zone. The quantification of waterborne parasite DNA was used to assess the potential reduction in parasite numbers associated with carcass removal.

METHODS

Study location.—Field studies occurred in the lower Klamath River basin below Iron Gate Dam (river kilometer [rkm] 307), which is a barrier to anadromous salmon passage (Figure 1). Sites included: the main stem of the Klamath River between Bogus Creek and the confluence of the Shasta River (rkm 284.6); Bogus Creek, the first tributary below Iron Gate Dam, which enters the Klamath River at rkm 306; Shasta River at the counting weir 0.8 rkm above the confluence; and the Trinity River, a major tributary of the Klamath River, where carcasses were collected between rkm 172 and rkm 177. Bogus Creek was divided into two reaches for sample collection and carcass removal (reach 1: from the mouth upstream 0.5 km to a counting weir; reach 2: from the weir upstream 1.5 km to a small waterfall, "Lower Falls").

Myxospores in salmon carcasses.—In 2008, the first 20–30 adult Chinook Salmon carcasses were sampled weekly at Bogus Creek regardless of their state of decomposition. In 2009–2012, only decomposed (moderate to extensive rating according to Baumsteiger and Kerby 2009) carcasses were sampled from Bogus Creek and the Klamath, Shasta, and Trinity rivers over a 4–5-week period in the fall. Fork length and sex (by gonad type observed during dissection) were recorded for each sample, and the intestine (small intestine [below the insertion of pyloric ceca] to rectum) was dissected from the carcass, placed into individually numbered plastic bags, and either refrigerated for 24 h or frozen prior to processing. The sample was weighed and then cut into 8–12-cm pieces, and an intestinal content sample (scraping) was obtained by grasping the intestine pieces with forceps and pushing the backside of a #21 scalpel blade, held at a 45° angle, along the outside of the intestine. The process was repeated several times until only the serosa to stratum compactum layers remained. The scraping subsample was weighed, diluted 25% with phosphate-buffered saline (PBS) at pH 7.4, poured into tubes, vortex mixed, and allowed to settle for 30 s before microscopic examination of a wet mount. Any scraping that weighed <1.0 g was diluted with 1.0 mL of PBS to provide enough sample material. A 100-µL aliquot was frozen for later quantitative PCR (qPCR) analysis. Duplicate 20-µL aliquots of this suspension were examined for the presence of *C. shasta* myxospores under 20× phase microscopy. Four hemocytometer counts on positive samples of wet mounts quantified myxospore concentration per milliliter (converted to per gram as 1 mL PBS weighed 1 g) of sample. This value multiplied by the scraping weight (g) provided the "myxospore per scraping" estimate. Given our limited detection sensitivity and other potentially infected tissues within a fish, we considered the myxospore per scraping value to represent the minimum spore load for a given fish. Prevalence of myxospore detection was determined as the number of myxospore positive samples per total sample.

As an alternative to performing polychaete infection experiments to ascertain the proportion of potentially infectious myxospores in a sample, we determined the percentage of intact (proxy for viable) myxospores. The hemocytometer sample was diluted in a 0.25% methylene blue dye solution using PBS (Hoffman and Markiw 1977). Intact spores could be differentiated from damaged spores because they are refractive to the dye whereas a spore with an incomplete cell membrane and/or broken valve appears blue. These samples were collected the day before processing and had not been frozen.

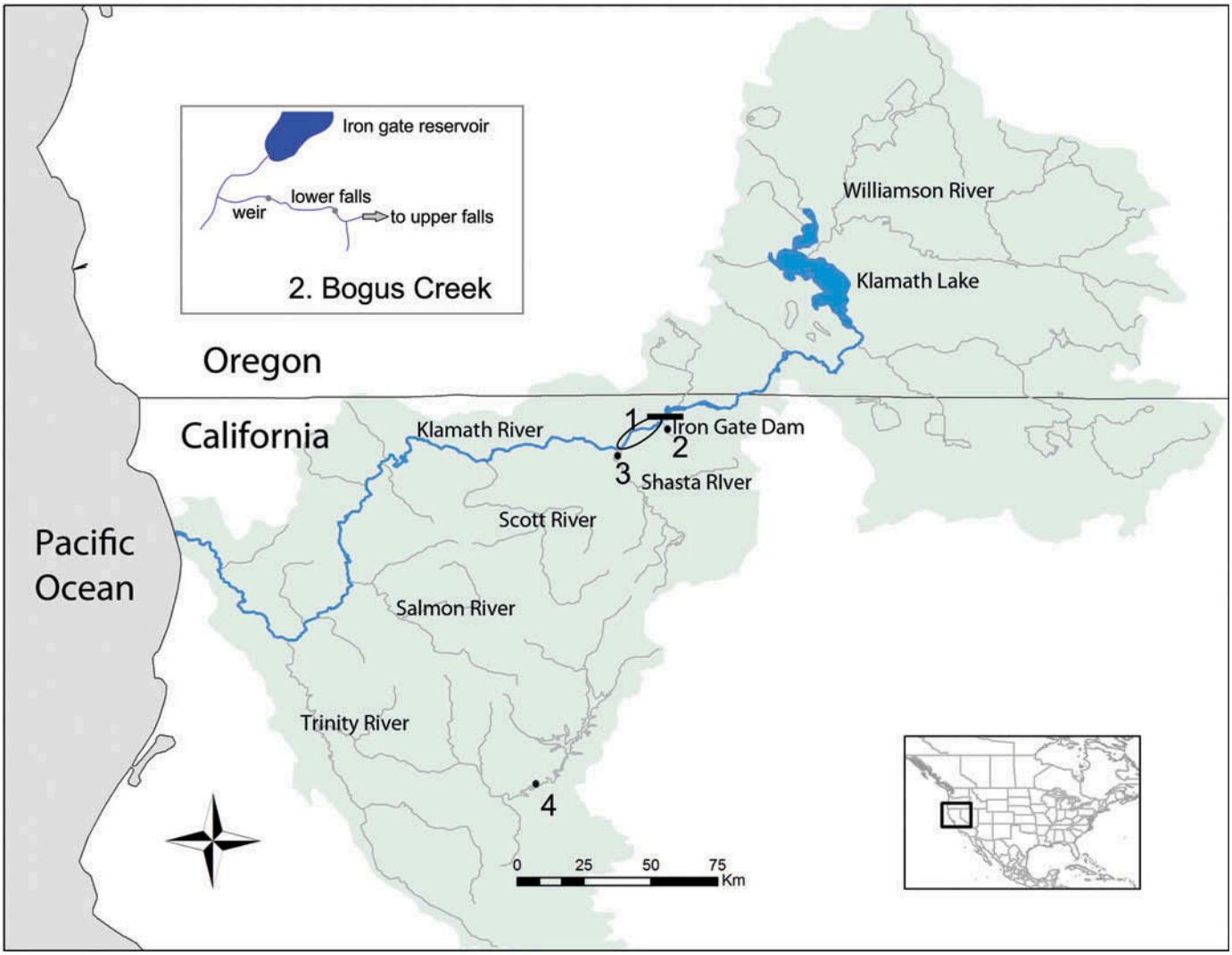

FIGURE 1. Sample sites on the Klamath River (1, between mouth of Bogus Creek and Shasta River), Bogus Creek (2, insert showing location of weir at top of reach 1), lower Shasta River (3), and Trinity River (4).

To estimate the prevalence of infection by all parasite stages in the visually negative samples, 6–27 scrapings, classified as myxospore "undetected," from each sample week per site were assayed by qPCR for *C. shasta* DNA. Scrapings were digested in 1 mL NucPrep digest buffer containing 1.25 mg/mL proteinase K (Applied Biosystems, Foster City, California) at 55°C for 2 h with constant shaking. A subsample of digested tissue homogenate diluted 1:33 in molecular biology grade water was extracted in a 96-well filter plate system (model 6100 Nucleic Acid PrepStation; Applied Biosystems) and stored at −20°C. The samples were tested for *C. shasta* 18S rDNA using a TaqMan Fam-Tamra probe and primers in an Applied Biosystems 7300 Sequence Detection System (Hallett and Bartholomew 2006). Reaction volumes of 30 µL, containing 5 µL of DNA template, were used under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Standards, extraction control, and no template control wells were included on each assay plate (Nichols et al. 2008). Detection thresholds were set at a relative fluorescence (ΔRn) of 10,000 fluorescent units, which occur at threshold cycle or quantification cycle (C_q) values of 38–39.

Carcass removal.—In 2008 and 2009, Yurok Tribal Fisheries biologists collected carcasses by hand within reach 1 of Bogus Creek daily over a 6-week period (October–late November). All carcasses were buried.

Density of parasite DNA in water and sediment.—Waterborne parasites in Bogus Creek were measured by molecular quantification of DNA in filtered water samples. Water samples were collected from two sites on Bogus Creek (downstream reach 1 and downstream reach 2) by Yurok and

Karuk tribal biologists from October 2008 to December 2009 during salmon in-migration and spawning. The rationale of the experiment was that the quantity of *C. shasta* DNA would not be the same or lower during the study period in the water samples from reach 1 compared with those from reach 2 if carcass removal in reach 1 reduced myxospore concentrations. Automated ISCO water samplers (Teledyne Isco, Lincoln, Nebraska) were programmed to collect 1 L of creek water every 2 h for 24 h into a large reservoir. From this 12-L composite, four 1-L samples were removed, filtered, and frozen and then sent to the laboratory (J. L. Bartholomew, Oregon State University, Corvallis, Oregon) for molecular processing (Hallett and Bartholomew 2006). For DNA extraction, the filter paper of samples collected in 2008 was cut (as per Hallett and Bartholomew 2006) whereas the filter of those collected in 2009 was dissolved in acetone (as per Hallett et al. 2012), and the final 2009 values were adjusted for comparison with the 2008 data. Samples were tested in duplicate wells and rerun if duplicates differed by more than 1 SE. Two positive controls (also assayed in duplicate) and negative controls (molecular biology grade water) were included in each plate. One sample from each site and time was tested for inhibition as per Hallett et al. (2012). When inhibition was detected, all cohort samples were diluted or repurified and reanalyzed. Based on reference samples with known numbers of myxospores, a C_q value of 35.1 equated to 1 myxospore/L. Samples in which no target DNA was detected were assigned a C_q of 42.

In response to measuring low numbers of waterborne parasites between October 2008 and December 2009, we collected substrate samples in December 2010 from Bogus Creek transects in reach 1 and reach 2 (one upstream and one downstream from the fishweir). On each transect, the substrate was isolated using bottomless buckets buried approximately 10 cm deep at three roughly equidistant points. The substrate was elutriated for 3 × 10 s by vigorous stirring with one hand. One 1-L water sample was collected from the middle of the bucket at the end of each elutriation period for a total of three 1-L samples. Carcasses were visible upstream from both transects and redds were avoided during sampling. In the laboratory, samples were centrifuged at 2,500 × *g* to separate the sediment and water fractions. Water and substrate fractions were quantified (total volume) and filtered (0.5 μm), and DNA was extracted (water sample protocol for water fractions and soil kit protocol for substrate fractions; QIAmp DNA Stool Mini Kit, QIAGEN) and tested for *C. shasta* by qPCR. Positive control samples were also prepared to determine the detection limit; sediment samples were collected from the Willamette River, Oregon, and spiked with myxospores harvested from laboratory fish (1, 5, 500, 5,000, 50,000, and 500,000 myxospores per sample, $n = 2$ sediment samples per dose).

Sentinel fish exposures.—Molecular (qPCR) analysis of water samples does not distinguish between actinospores, the infectious stage for fishes, and myxospores, the infectious stage for polychaetes. Thus, to determine the spore stage of the parasite DNA detected in creek water samples, we conducted concurrent sentinel fish exposures using highly susceptible Rainbow Trout *O. mykiss* (Bjork and Bartholomew 2009) below the weir in reach 1. Forty-five Rainbow Trout from Roaring River were held in a 302-L aluminum live box for 13 d (during which they were checked and fed weekly) on three occasions in 2008: September 24, October 8, and October 22. Fish were then transported to the wet laboratory at the California–Nevada Fish Health Center for observation and sampling. Fish were held in a 750-L observation tank at a water temperature similar to that of Bogus Creek (15°C) for 20 d and then euthanized with an overdose of tricaine methanesulfonate (MS-222), measured (FL), weighed and examined for signs of disease. The intestinal tract and kidney were removed and sampled for qPCR (True et al. 2012) and histology. Five unexposed fish were sampled at the start of each exposure.

Data analyses.—Paired *t*-tests (PROC TTEST, SAS version 9.3; SAS, Cary, North Carolina) were used to test for differences in parasite DNA levels above and below the carcass removal reach. SigmaPlot12 (Systat software 2010) was used to perform linear regression, one-way ANOVA, and Mann–Whitney rank-sum and *t*-tests.

RESULTS

Myxospores in Chinook Salmon Carcasses

As observed in this multiyear data set, minimum detection levels observed in the wet-mount screening method was approximately 1,000–3,000 myxospores per scraping and was likely influenced by a random distribution at low concentrations. Serial dilution (with both PBS and spore-negative suspension) of high-spore concentration samples followed by wet-mount screening showed a detection range of 118–400 spores/μL. Scraping weight averaged 26% of the intestine sample. Annual prevalence of myxospore detection ranged from 22% to 52% per sample site (Table 1). Spore concentration ranged from 394 to 14.7 million myxospores per scraping with a 7.3% (44 of 602) prevalence of "high" spore contributors (>500,000 spores). These high-spore carcasses contributed between 76% and 95% (mean 89%) of the estimated annual site spore total. When the three outlier samples that had ≥13 million spores were removed from the data set, total myxospore counts of the collection groups ranged from 2.5 to 21.3 million myxospores per scraping (Table 1). Prevalence of *C. shasta* DNA in visually negative samples ranged from 45% to 87%. Detection of DNA in the absence of visible myxospores indicated the presence of either low myxospore numbers or nonspore parasite stages within the majority of intestine samples (Table 1).

In the 2008 data set for Bogus Creek, decomposed adult samples had significantly higher myxospore loads (median, 182,109) than did fresh (median, 30,656) carcasses (Mann–Whitney rank-sum test, $t = 136$, $n = 30$, $P = 0.036$). Decomposed carcasses were collected after 2008. Myxospore quantity was not related to carcass sex (Mann–Whitney rank-

TABLE 1. The prevalence of myxospore detection in the intestines of Chinook Salmon carcasses (*n* myxospore positive/total sample [%]), prevalence of samples >500,000 spores, total myxospores in seasonal sample (spores × 10^6), and prevalence of *C. shasta* DNA detection determined by PCR in samples identified as "myxospores undetected" of fall-run Chinook Salmon carcass intestine samples from Bogus Creek, Shasta River, and Klamath River in 2008–2012.

Site sampled	Year sampled	Prevalence of myxospores (*n*/total [%])	Prevalence with >500,000 spores (*n*/total [%])	Number of spores (× 10^6)	Prevalence of parasite DNA detection (*n*/total [%])
Bogus Creek	2008	30/100 (30)	7/100 (7)	38.7[a]	5/11 (45)
	2009	14/65 (22)	3/65 (5)	11.0	20/24 (83)
	2011	13/60 (22)	1/60 (2)	6.0	5/6 (83)
Shasta River	2009	32/61 (52)	7/61 (11)	13.4	10/15 (67)
	2011	23/60 (38)	4/60 (7)	9.3	6/7 (86)
	2012	29/80 (36)	7/80 (9)	21.3	22/27 (81)
Klamath River	2009	14/56 (25)	6/56 (11)	11.8	11/18 (61)
	2011	20/55 (36)	7/55 (13)	22.8[b]	6/7 (86)
	2012	14/65 (22)	2/65 (3)	2.5	20/23 (87)
Total		189/602 (31)	44/602 (7)	136.8	105/138 (76)

[a]10.1 million if outlier sample >13 million excluded.

[b]9.8 million if outlier sample >13 million excluded.

sum test: $t = 169$, $n = 30$, $P = 0.966$). No trend in myxospore quantity was detected in other collection groups containing similar carcass numbers of both sexes for date of death (collection) or age (based indirectly on FL) (Table 2).

The effect of residence time for Chinook Salmon prior to postspawning death on myxospore production is exemplified by the larger quantity of myxospores observed in Trinity River spring-run salmon than in the fall-run salmon in 2012. Prevalence of myxospore detection in Trinity River spring-run salmon (38 of 60, 63%) was similar to that of the fall-run salmon (36 of 59, 61%) carcasses; however, spring-run Chinook Salmon had a higher prevalence of carcasses (30% compared with 19% for fall-run salmon) containing >500,000 myxospores. If one fall-run salmon carcass outlier, containing an estimated 61.5 million myxospores per scraping, is removed from the data set, the total myxospore estimate from spring-run Chinook Salmon (81.3 million) was 2.4 times greater than that for fall-run salmon (33.9 million). Freshwater residency of the Chinook Salmon for both runs was estimated from California Department of Fish and

TABLE 2. Relationship of myxospore quantity (spores) of positive samples to carcass FL (surrogate for age), collection date (surrogate for date of death), and sex in data sets with similar numbers of male and female Chinook Salmon from 2008 Bogus Creek (BC), 2009 combined Klamath and Shasta rivers (KR&SR), and 2012 combined spring- and fall-run salmon in the Trinity River (TR): linear regression equation and coefficient of determination (R^2) for FL and date of collection; Mann–Whitney rank-sum test results (*P*-value) for myxospore quantity between sexes (*N* = sample number). ND: no data as difference in spring- and fall-run Chinook Salmon prohibited combining the data sets.

Data set	*N*	FL	Date of collection	Difference between sexes
2008 BC	100	Spore = −954603.711 + (19336.070 × FL) $R^2 = 0.0127$	Spore = −4811785249.815 + (18255.114 × date) $R^2 = 0.00565$	$P = 0.966$, $n = 30$
2009 KR&SR	117	Spore = 364166.300 + (8131.147 × FL) $R^2 = 0.0176$	Spore = 34959693971.280 − (14239.309 × date) $R^2 = 0.0364$	KR, $n = 14$ $P = 0.289$ SR, $n = 32$ $P = 0.205$
2012 TR	120	Spore = 4483423.998 − (38045.833 × FL) $R^2 = 0.00182$	ND	Fall, $n = 37$ $P = 0.353$ Spring, $n = 38$ $P = 0.872$

Wildlife (CDFW) 2012 Klamath River estuary angler harvest and carcass data (S. Borok and A. Hill, CDFW, personal communication). We estimated accumulated temperature units (ATU = summation of mean daily temperature, °C) from mean weekly water temperature exposure per reach (U. S. Geological Survey and California Department of Water Resource gauge data). Spring-run Chinook Salmon spent almost twice as long in freshwater (mean, 119 d; range, 105–123 d) as fall-run salmon (mean, 63 d; range, 49–77 d). Primarily due to a longer holding period in the spawning reach, spring-run salmon experienced 46% to 59% higher ATUs (mean ATU = 1,581°C for peak entry; range, 1,466–1,716°C) than fall-run salmon (mean ATU = 846°C; range, 675–1012°C).

Myxospore Viability

Myxospore dye exclusion (spore integrity was used as a proxy for spore viability) was examined over time from adult carcass samples of Chinook Salmon. Nineteen nonfrozen samples stained with 0.25% methylene blue were examined 24 h after collection and a second time at 32–53 d postcollection (dpc). The percent intact myxospores (dye excluded) ranged from 7% to 100% 24 h after collection (Figure 2); there was a trend towards lower percentages of intact spores in higher-concentration samples (percent intact spores = 83.180 − 12.872 × million myxospores per scraping, $R^2 = 0.425$). When the refrigerated samples were assessed a second time at 32–53 dpc, the percentage of intact spores declined sharply to 0–18% in samples where the 1-dpc value was <85% (Figure 3).

Carcass Removal and Parasite DNA in Water and Sediment

Between October 10 and November 24, 2008, 907 Chinook Salmon carcasses were removed from reach 1, which represented approximately 19% of the total run past the counting weir. No *C. shasta* infection was detected by qPCR or histological examination of sentinel Rainbow Trout exposed in reach 1, indicating that actinospores were not present in Bogus Creek during the study period. Waterborne parasite levels ranged from undetected to approximately 5 spores/L. The highest levels were detected during the peak spawning period of late October through mid-November (Figure 4a). No significant difference in DNA quantity was detected between reaches 1 (carcasses removed) and 2 (carcasses not removed) (*t*-test: $t = 0.37$, df = 13, $P = 0.72$).

Between October 12 and November 19, 2009, 1,799 carcasses were removed from reach 1, which represents approximately 23% of the total return of salmon to Bogus Creek. Similar to 2008, levels increased during spawning and peaked at 1 spore/L in late October (Figure 4b). No significant difference in DNA quantity was detected between reaches 1 and 2 (*t*-test: $t = 0.80$, df = 12, $P = 0.44$). *Ceratonova shasta* DNA was detected in December 2010 in field-collected substrate fractions that corresponded to levels of five myxospores per sample in positive controls. Parasite DNA was only detected in the sediment fractions, not the water fractions. In positive

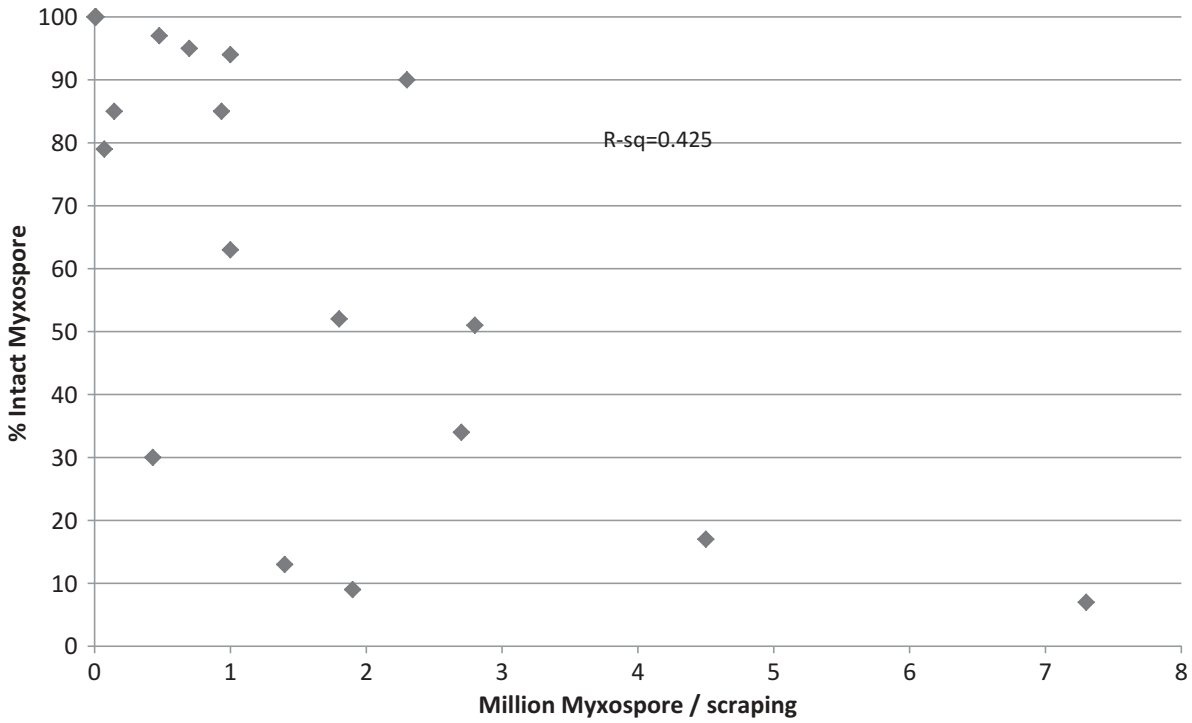

FIGURE 2. The relationship between million myxospores per scraping and percent intact (dye exclusion) myxospores at 1 d postcollection.

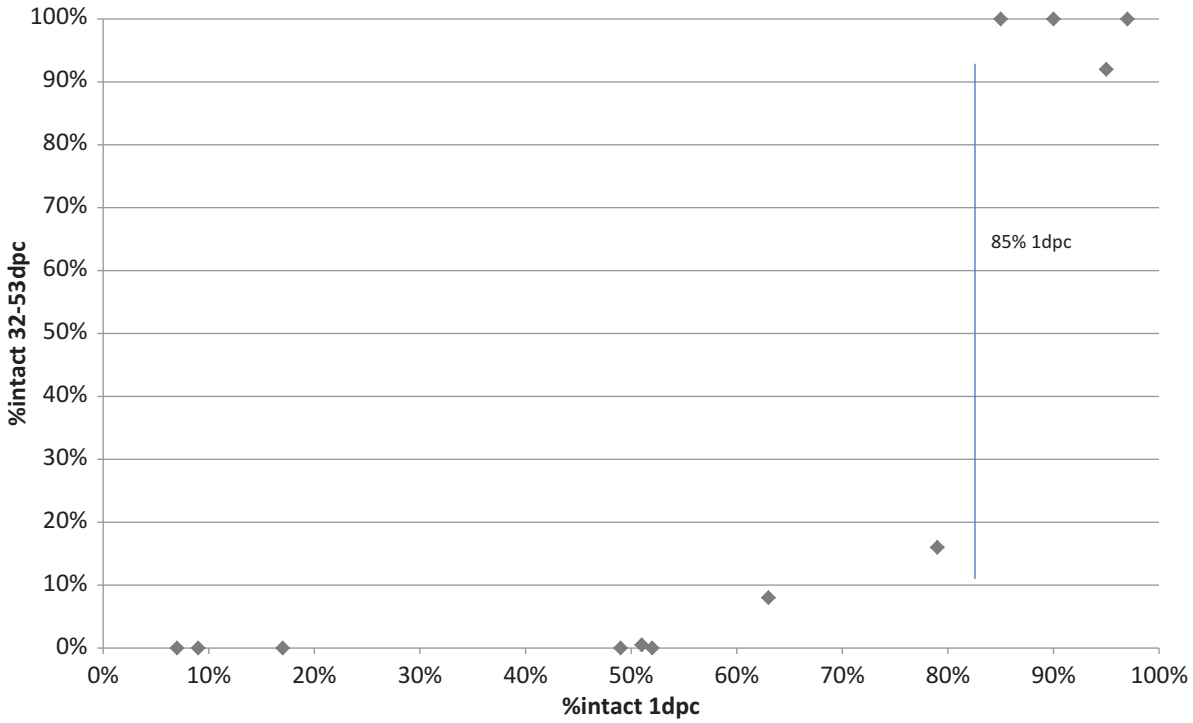

FIGURE 3. The relationship of percent intact (dye exclusion) myxospores at 1 d postcollection (dpc) and after 32–52 d of storage at 4°C.

sediment controls, *C. shasta* DNA was detected at five spores per sample and above but not as low as one spore.

DISCUSSION

Host–parasite dynamics in a variable riverine environment are complex. To better understand the contribution of myxospores from spawned adult salmonids during the *C. shasta* life cycle in the Klamath River system and potential transmission to the invertebrate host, we assessed the prevalence of infection and myxospore production and viability (dye exclusion) in free-ranging Chinook Salmon hosts. We also explored the potential reduction in parasites available to infect polychaetes through the removal of salmon carcasses and sampling of environmental *C. shasta* DNA.

The prevalence of *C. shasta* infection (all vertebrate host stages) was high (62–91%) among adult Chinook Salmon stocks in the Klamath River basin, and myxospores were found predominately in decomposed carcasses rather than in recently postspawned fish. Intestinal infection, at both the trophozoite and presporogonic stages of the parasite, was common among spawned fall-run adult Chinook Salmon sampled between 2005 and 2007 at Iron Gate Hatchery (U.S. Fish and Wildlife Service, unpublished data). Myxospores were detected in far fewer of these adults. In 2005, histological sections from tissues of spawned Chinook Salmon adults showed an 80% incidence of infection at only the presporogonic stages (16 of 20 sections). In 2006, *C. shasta* myxospores were observed in only 1 of 60 intestinal scraping samples, while *C. shasta* DNA was detected in a subset by qPCR in 12 of 20 (60%) scrapings. A similar effort by Oregon State University and CDFW to survey spawned fish from the Iron Gate Hatchery by qPCR yielded 70% and 85% detection rates of *C. shasta* DNA in 2005 and 2006, respectively. In 2007, *C. shasta* myxospores were observed in the intestinal scraping of only 4% (6 of 166) of spawned adult salmon from the Iron Gate Hatchery and none of the 154 adult salmon sampled in the Klamath River estuary soon after freshwater entry (Ryan Slezak, Humboldt University, personal communication). These data indicated that myxospores were found primarily in carcasses from adult salmon that had senesced and died days after spawning. It is possible that the pansporocyst stage (presporogonic) responds to changes in the microenvironment associated with decomposing tissue (e.g., lytic enzymes, oxygen and pH changes) to complete sporogenesis (see also Kent et al. 2014). Myxospore increase in fish carcasses postmortem has also been observed in another Pacific Northwest river, the Williamette River (Kent et al. 2014).

While myxospores occurred in 22–52% of the fish carcasses examined from the Klamath River basin, a much smaller subset (<10%) provided on average 89% of the potential spore input. These data indicated the strong influence of only a few high spore contributors to the potential number of myxospores released into a body of water. Our observation of an inverse relationship between carcass spore quantity and viability (dye exclusion) calls into

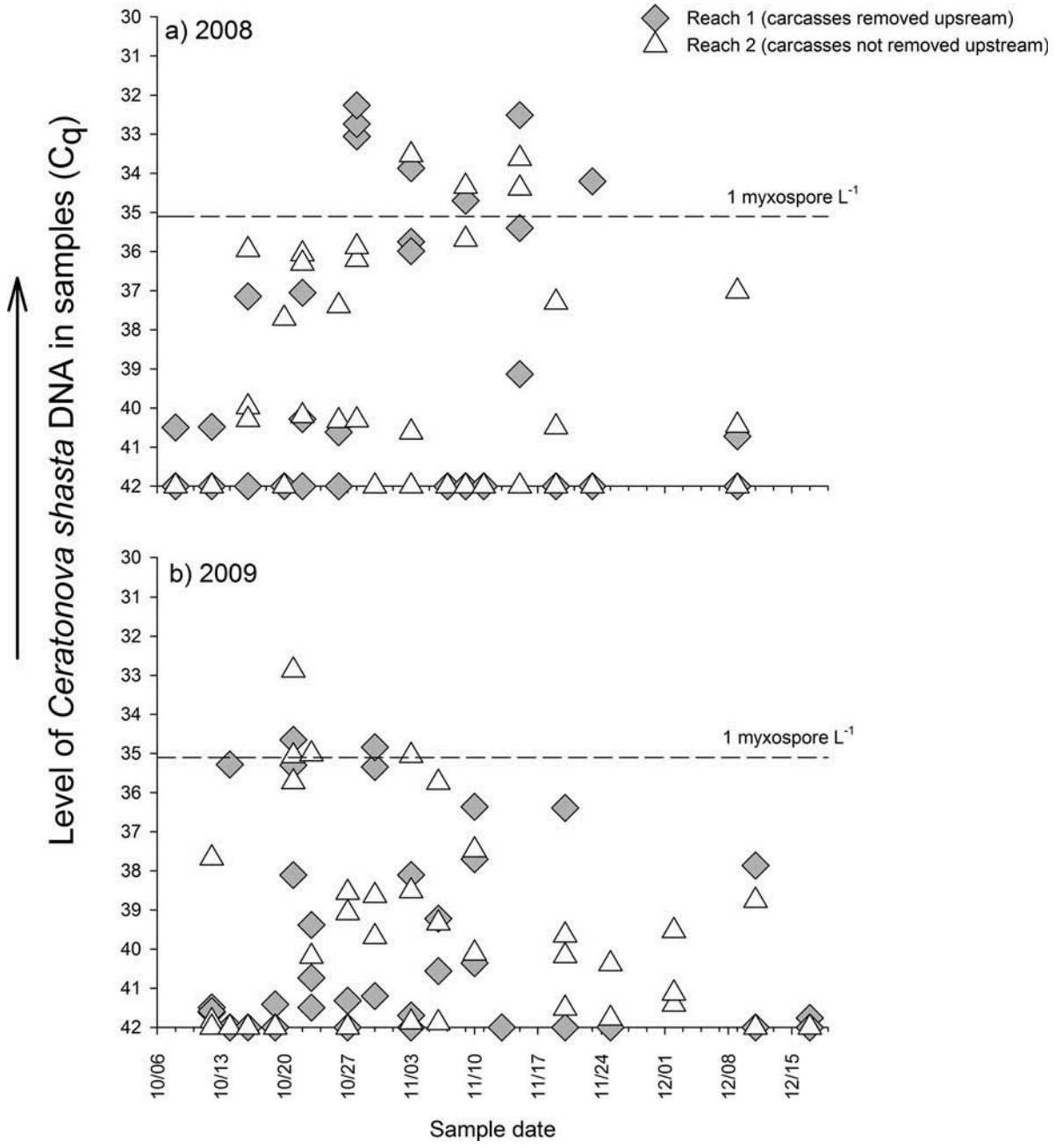

FIGURE 4. Mean *Ceratonova shasta* DNA quantity (C_q) in water samples collected from the mouth of Bogus Creek (bottom of reach 1) and counting weir (bottom of reach 2, top of Chinook Salmon carcass removal reach) in (**a**) 2008 and (**b**) 2009. Each data point represents one of three replicate 1-L water samples collected at that time. The quantity of DNA data ranged from zero in samples in which no target DNA was detected (assigned C_q of 42) to approximately 5 spores/L.

question the actual contribution of the high-spore carcasses towards transmission of the parasite. High myxospore production would be associated with extensive intestinal necrosis (Bartholomew et al. 1989), which could impair spore development by limiting nutrition and interaction with lytic enzymes. Further work on this aspect, such as verification of spore viability with polychaete infection experiments, is needed prior to using prevalence data for valid life cycle modeling (Ray et. al. 2010). The positive ATU relationship on myxospore quantity suggests that freshwater resident time of an infected adult salmonid population (e.g., spring-run Chinook Salmon) will increase their myxospore input to the river. Wagner et al. (2005) reported the positive influence of ATU > 450°C on the severity of infection by the myxosporean parasite, *Parvicapsula minibicornis*, on adult Fraser

River Sockeye Salmon *O. nerka* but did not discuss myxospore production.

Although parasite levels in water samples were relatively low (compared with main-stem sites; see Hallett and Bartholomew 2006; Hallett et al. 2012), they appeared to increase slightly during the spawning period, which can be attributed to myxospores released from postspawned fish rather than actinospore release since highly susceptible sentinel fish did not become infected.[1] Also, real-time PCR analysis of water filters has detected over 20-fold higher actinospore numbers in river water (Hallett and Bartholomew 2006). It is not surprising that water sampling efforts detected low quantities of *C. shasta* DNA given the large volume of Bogus Creek flow during the testing period and resulting possibility of myxospore transport. For example, if the 2008 prevalence of infection for low to moderate (23%) and high spore load (7%) is extrapolated to the 4,566 adults that returned to Bogus Creek (Klamath River basin megatable: M. Knechtle, CDFW, personal communication), the number of myxospore-positive carcasses would be estimated at 1,050 and 320, respectively. The median spore load of the low to moderately infected group was 40,500 and was 2,159,500 for the high group. A summation of the product (median spore load × number of infected fish) yields an estimate of 732,751,680 myxospores present in Bogus Creek. If we assume that myxospore release and transport are constant over the 56-d water sampling period, then we would expect approximately 13,085 myxospores to be released into the creek each day. On average, creek discharge during October–December 2008 was 48,931,680 L/d (566 L/s: M. Knechtle, CDFW, personal communication), which yields an estimate of 0.27 spores$\cdot L^{-w} \cdot d^{-w}$. This estimate is consistent with measured levels of DNA in the creek water. Thus, although we observed a high amount of variability in DNA levels that precluded the detection of a statistically significant increase in DNA levels, the increase in DNA levels from mid-October until late November may in fact be real. We speculate that winter rain events act to transport sediment-bound myxospores into the river and may play an important role in the annual infection cycle. The detection of myxospores in Bogus Creek sediment supports this hypothesis. Despite dilution effects of river flow and patchy polychaete distribution on myxospore transmission, actinospore release from the invertebrate host has been documented for many years in the Klamath River indicating an efficient annual transmission (Hallett and Bartholomew 2006; Stocking et al. 2006; Hallett et al. 2012). Similar to the 2008 Bogus Creek myxospore production estimate, salmon carcasses in the Klamath and Shasta rivers could provide billions of myxospores for potential transmission to polychaetes within the infectious zone on an annual basis.

We did not observe an obvious trend of spore reduction (consistent, similar, or reduced DNA concentration between upstream and downstream removal reaches) in Bogus Creek. However, our ability to evaluate different levels of carcass removal effort was constrained by the resolution and detection threshold of the water sampling technique (i.e., at parasite levels < 1 spore/L of river water). Irrespective of this analytical limitation, removal of Chinook Salmon carcasses was deemed inefficient for reducing myxospore transmission given the high number of myxospore-contributing carcasses, lack of spatial or temporal focus to improve efficiency of carcass removal, and problematic logistics (intensive labor, worker safety concerns, carcass disposal costs, low recovery from deep reaches, and controversy on nutrient removal from the river). Mitigation efforts will need to look to other management options such as the reduction of polychaetes or implementation of increased flows (Alexander et al. 2014).

ACKNOWLEDGMENTS

We sincerely thank the following individuals for collecting intestine samples: Bogus Creek collection (Yurok Fisheries): Josh Strange, Troy Fletcher Jr., and Albert Marlusser; Klamath River (Arcata Fish and Wildlife Office): Steve Gough, Ernest Chen, Tony Heacock, Aaron Johnson, Mark Magneson, Dan Menten, Amanda Piscitelli, Mandy Proudman, Ryan Slezak, and Katrina Wright. Access to carcasses was provided by Morgan Knechtle in the Shasta River and by Andy Hill in the Trinity River. Access to spawned adults was provided by the staff at the Iron Gate and Trinity River hatcheries. Ken Nichols assisted with laboratory processing and Adam Ray with water and sediment DNA sampling. Partial support for this work was derived from grants with the Pacific States Marine Fisheries Commission, U.S. Bureau of Reclamation, and National Oceanic and Atmospheric Administration. The mention of specific products does not constitute endorsement by the U.S. Fish and Wildlife Service.

[1] For many years, highly susceptible Rainbow Trout have been used to detect *C. shasta* in waterways. However, subsequent to our conducting the Bogus Creek sentinel fish exposures with out-of-basin Rainbow Trout, different genotypes of *C. shasta* were identified that are associated with different salmonid host species (Atkinson and Bartholomew 2010). For example, genotype I causes mortality in Chinook Salmon, whereas genotype II can be fatal for Coho Salmon *Oncorhynchus kisutch* and Rainbow Trout. Thus, sentinel trout would only detect a subset of the potential parasite population in Bogus Creek (genotype II but not genotype I). So the statement that the parasite eDNA in Bogus Creek water samples was strictly myxospore derived rather than actinospore derived is only partially supported by the negative infection response of the sentinel trout. We cannot completely rule out the presence of *C. shasta* genotype I actinospores without using Chinook Salmon sentinels. However, we are still confident that the majority of the eDNA is myxospore derived since we found high numbers of these spores in the local salmon (carcasses) and the habitat in that creek is regarded as unsuitable for the polychaete host that produces the actinospore stage.

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