Density of the Waterborne Parasite *Ceratomyxa shasta* and Its Biological Effects on Salmon

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The myxozoan parasite *Ceratomyxa shasta* is a significant pathogen of juvenile salmonids in the Pacific Northwest of North America and is limiting recovery of Chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon populations in the Klamath River. We conducted a 5-year monitoring program that comprised concurrent sentinel fish exposures and water sampling across 212 river kilometers of the Klamath River. We used percent mortality and degree-days to death to measure disease severity in fish. We analyzed water samples using quantitative PCR and Sanger sequencing, to determine total parasite density and relative abundance of *C. shasta* genotypes, which differ in their pathogenicity to salmonids. We detected the parasite throughout the study zone, but parasite density and genetic composition fluctuated spatially and temporally. Chinook and coho mortality increased with density of their specific parasite genotype, but mortality-density thresholds and time to death differed. A lethality threshold of 40% mortality was reached with 10 spores liter^{-1} for Chinook but only 5 spores liter^{-1} for coho. Parasite density did not affect degree-days to death for Chinook but was negatively correlated for coho, and there was wider variation among coho individuals. These differences likely reflect the different life histories and genetic heterogeneity of the salmon populations. Direct quantification of the density of host-specific parasite genotypes in water samples offers a management tool for predicting host population-level impacts.

T he Klamath River (KR) spans 423 river kilometers (Rkm) from south-central Oregon to northwestern California, and its environmental profile has been severely altered by anthropogenic activities (21). For anadromous fish, over one-third of the river has been rendered inaccessible by a series of dams that regulate flow and reallocate water, and the remainder is a challenging habitat due to decreased flows and high summer water temperatures (21). Endemic salmonid (salmon and trout) populations have declined drastically over the past 2 centuries as a result of these abiotic burdens and the emergence of pathogen-induced diseases.

For the past decade, ceratomyxosis (enteronecrosis) has been regarded as the major cause of mortality in fall Chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon (10, 13). Ceratomyxosis is caused by the myxozoan parasite *Ceratomyxa shasta*, which is present throughout the Pacific Northwest, but its negative impacts on native salmonids are most thoroughly documented in the KR. Severe ceratomyxosis in outmigrating juvenile fish contributes to reduced recruitment and adult returns (13, 29).

Salmon become infected by microscopic *C. shasta* actinospores, which are released from freshwater polychaetes into the water column (6), enter the fish host's gills, and migrate through the circulatory system to the intestine where they develop into myxospores (8). Salmon in the KR have coevolved with *C. shasta* and are relatively resistant to infection (4), and thus, the losses observed recently are atypical (5, 20). The host-parasite balance has likely been upset by perturbations in water flow, sedimentation, and temperature associated with anthropogenic activities. Of these factors, the effect of temperature on disease progression in salmonids is the best studied: as temperature increases, more fish succumb to disease and they do so faster (25, 31). However, temperature variation alone is insufficient to explain differences in fish mortality, and exposure dose is posited to be an important contributing parameter (11, 25).

Measuring the exposure dose in the natural environment pres-

ents challenges. Juvenile salmonids may be exposed to *C. shasta* actinospores for many weeks during rearing and subsequent outmigration. These fish may become infected at low doses but do not become diseased unless overwhelmed by spores (12). A water-sampling method developed to detect and quantify waterborne stages of *C. shasta* was used at locations in the upper and lower KR to compare parasite densities with mortality of nonindigenous rainbow trout (*O. mykiss*) (15, 28). No consistent relationship between these two measures was found; although total spore density was similar at several field sites, fish mortality differed. This puzzling observation was later explained by the discovery of several *C. shasta* internal transcribed spacer region 1 (ITS1) genotypes that differ in host-associated mortality (2, 3). In particular, genotype I causes mortality in Chinook, whereas genotype II is pathogenic for coho and rainbow trout. Thus, water sample analysis must consider the host-specific genotype contribution to the total parasite abundance.

Accordingly, we collected water samples during sentinel exposures of Chinook and coho salmon at four index sites in the lower KR (Fig. 1) from 2006 to 2010. We used quantitative PCR (qPCR) to estimate parasite density (number of *C. shasta* spores liter^{-1} of filtered river water) (15) and directly sequenced DNA in positive samples to determine the relative abundance of *C. shasta* ITS1 genotypes (2, 3). We compared parasite density with sentinel fish
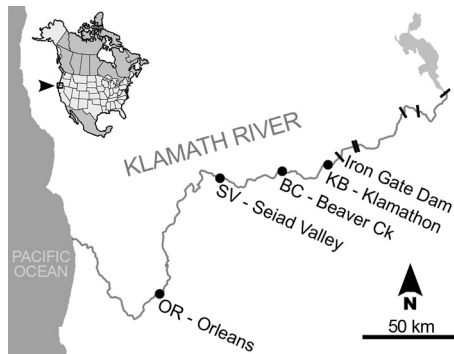
FIG 1 Locations of index sites for sentinel fish exposures and water sampling in the lower Klamath River, CA. Bars denote dams.

mortality and constructed logistic regression models to determine if levels of parasite (genotype specific) could be used as a tool to predict mortality in salmon.

MATERIALS AND METHODS

Study sites. We established four index sites in the main stem of the lower KR (downstream of Iron Gate Dam, CA) that span 212 Rkm: Klamathon Bridge (KB), 302 Rkm from the mouth; Beaver Creek (BC), 258 Rkm; Seiad Valley (SV), 207 Rkm; and Orleans (OR), 90 Rkm (Fig. 1).

Sentinel fish exposures. KR Fall Chinook and coho salmon from the California Department of Fish and Game, Iron Gate Hatchery, were held at each index site in separate cylindrical mesh cages for 3 days each month from April to October 2006 to 2010. Both species were not held at all sites in all months; exposure dates, locations, and fish numbers are detailed in Table S1 in the supplemental material. We timed the field exposures to overlap outmigration of juvenile salmonids and the release of hatchery salmon and to avoid lethal elevated water temperatures (>22°C in July/August) and associated summer pathogens such as *Flavobacterium columnare*, the cause of columnaris disease. Nonindigenous rainbow trout from Roaring River Hatchery (Scio, OR, Oregon Department of Fish and Wildlife) with known high susceptibility to *C. shasta* were held alongside the native species as positive controls. Unexposed fish of each species were negative controls. River temperature was measured with a submerged HOBO temperature recorder (Onset Computer Corporation, Pocasset, MA).

After exposure, the fish were transported in aerated coolers to the John L. Fryer Salmon Disease Laboratory (SDL), Oregon State University (OSU). Fish were given preventative treatments for columnaris during transportation to the SDL and for external parasites within ~1 week post-exposure (see reference 28). Each exposure group was held in a 25- or 100-liter tank (depending on fish size) and received either ambient (13°C) specific-pathogen-free well water or heated (16 to 20°C) well water to reflect current river water temperature (see Table S2 in the supplemental material for specifics). Fish were monitored daily for clinical disease signs for approximately 60 (2009 and 2010) to 90 (2006 to 2008) days postexposure. Moribund fish were euthanized with an overdose of MS222 (tricaine methanesulfonate; Argent Laboratories). A posterior intestinal sample was obtained by insertion of a sterilized metal loop and then examined in wet mount for mature parasite stages (myxospores) for up to 3 min using bright-field microscopy. Fish were considered visually positive for *C. shasta* if myxospores were observed. At termination, remaining fish were killed with MS222.

Two measures of severity of infection were used: percent mortality, which was calculated as (number of fish that died during holding that were visually positive for *C. shasta* divided by the total number of fish that had survived the first 5 days postexposure) × 100, and mean days to death, which was the geometric mean of all days with *C. shasta*-positive mortalities within the holding period. Percent prevalence of infection was not determined because Chinook can clear the infection within 60 days and

mortality is more relevant to population-level impacts. Fish that died from other causes were not included in the analyses.

Water samples. (i) Collection. Four 1-liter water samples were collected twice, near the cages, when fish were placed in the water and ~72 h later, when the fish were removed. Collection either was manual (2006 and 2007) or was done with an automated ISCO 3700 portable sampler (Teledyne) (2008 to 2010). The ISCO device sampled 1 liter every 2 h (with a single purge cycle) for 24 h into a reservoir from which four 1-liter homogenous samples were taken manually. Samples were held on ice or refrigerated until filtering, within 24 h of collection. Each 1-liter sample was filtered through a 5-μm-pore-size nitrocellulose membrane filter using a vacuum pump, and the filter was folded to fit into a 2-ml microcentrifuge tube and frozen. Samples containing considerable particulate matter were divided for filtering and then recombined during the extraction process (during application to the column).

(ii) Extraction. From 2006 to 2008, the filter disc was cut into strips prior to lysis and extraction (15). From 2009, an acetone dissolution protocol (modified from reference 1) replaced cutting: filters within each microcentrifuge tube were air dried (open lid) overnight, 1.5 ml of acetone was added, the tubes were then vortexed several times until the filter dissolved (~30 min) and then centrifuged, and the supernatant (except ~100 μl covering the pellet) was discarded. The acetone step was repeated to ensure complete dissolution of the filter and liberation of captured material, and then the sample was centrifuged and the supernatant was removed as described above. To each sample, 750 μl 95% ethanol was added and the mixture was vortexed to suspend the pellet, and then the samples were centrifuged and the supernatant was removed as described above. Samples were air dried overnight and then either extracted or stored at −20°C.

DNA was extracted and purified using a Qiagen DNeasy blood and tissue kit following the protocol for animal tissues with the following modifications: after addition of buffer ATL, the contents were mixed well to ensure suspension of the pellet, the RNase step was retained, an extra wash with buffer AW2 was added, and DNA was eluted in 2 washes of 60 μl buffer AE with 5 min incubation at room temperature. Extracted samples were stored at −20°C until assessed by qPCR. Generally, 3 of the 4 field water sample replicates were processed.

(iii) Assessment of protocol changes. The original and modified filter-processing methods were compared using three sets of water samples. (i) In May 2009, 7 1-liter samples were collected and filtered from the Willamette River, Corvallis, OR, where *C. shasta* is endemic; three filters were cut, and four were dissolved; each sample was then run in quadruplicate on a qPCR plate. (ii) Eight 1-liter samples were collected from above a laboratory population of *C. shasta*-infected polychaetes; half the filters were cut and half were dissolved; extracted DNA was assessed in duplicate wells on a qPCR plate. (iii) Additional spiked samples consisted of two filters spiked with one actinospore and another two filters spiked with 1 myxospore each; one from each pair was cut and the other was dissolved; they were assayed in duplicate qPCR wells. The average qPCR quantification cycle (C_q) values for each data set were compared.

(iv) qPCR. The density of *C. shasta* spores in each 1-liter water sample was determined using a TaqMan-based qPCR (15) and TaqMan gene expression master mix (Applied Biosystems) in an ABI Prism 7000, 7300, or 7500 sequence detection system and ABI MicroAmp AE optical 96-well reaction plates with 50 cycles of standard thermal cycling conditions. Samples were assayed in duplicate on a plate, and the data were averaged. Samples were considered positive if both wells fluoresced. Samples were reanalyzed if C_q values for their duplicate wells differed by more than 1 C_q or if one well was undetected. Undetected samples (both wells) were designated a C_q of 42, which represented <1 spore liter^{-1}, to enable their inclusion in analyses.

Each plate had 4 wells with only molecular-grade water as a negative control and 4 wells of known infected sample or synthetic template as a positive control. Reference samples were prepared whenever the pre-qPCR methodology was modified (e.g., introduction of filter dissolution)

and were reanalyzed when the qPCR was altered (e.g., when the master mix changed). These samples were processed identically to field samples but were spiked preextraction with known numbers of parasites (usually 1 myxospore). Cq values were translated to number of spores liter^{-1} using a standard curve (15).

A TaqMan exogenous internal positive control (IPC; AB) was used to assess inhibition in one of three field sample replicates. The IPC was incompatible with *C. shasta* in a multiplex assay (17); thus, a separate inhibition assay with 1 μl IPC probe/primer mix (0.5×) and 0.2 μl IPC DNA (0.5×) per 20-μl reaction mixture (plus 1 μl sample DNA) was required. Samples were run in duplicate and considered inhibited if fluorescence was retarded compared to a molecular-grade water sample, and Cq values for the other replicates were adjusted by the Cq difference to compensate. Samples with IPC values retarded by more than 2 Cq were diluted 1:10 and rerun, and their final Cq value was adjusted for dilution.

To compare data across years, Cq values were calibrated to 2008 values, based on reference samples with known numbers of spores.

(v) Genotyping of *C. shasta*. The ITS1 genotype composition of *C. shasta* in qPCR-positive water samples collected during the sentinel fish exposures was determined using PCR and Sanger sequencing, based on the protocols developed previously (2, 3), which we modified for difficult samples. Sequencing of all three 1-liter sample replicates per site and time was attempted.

We improved the genotyping PCR assay over the study period. Initially, water samples were diluted 1:10 (to reduce inhibition) and genotyped with two consecutive rounds with primers Cs1482F and NC13R (2). The specificity of the assay was improved with substitution of reverse primer CsgenR1 (3). Cycle parameters for these assays were 95°C for 2 min and then 35 cycles of 94°C for 20 s, 59°C for 30 s, 72°C for 1 min, followed by 72°C for 10 min. To address problems with gel smearing and inhibition associated with water samples, we developed a fully nested PCR assay: first-round primers Cs1 (22) and NC13R generated ~1,800 bp of SSU/ITS1 rRNA, and then second-round primers Cs1482F and CsgenR1 (3) generated ~450 bp of the ITS1 genotyping region. PCR chemistry and instrument were identical to those described previously (3), but note that the bovine serum albumin concentration was 250 μg/μl rather than 25 μg/μl, which was listed incorrectly in the previous report (3). The PCR program was modified for the larger first-round fragment: the first round was 95°C for 2 min, and then 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, followed by 72°C for 10 min; the second round was the same as that in the two-round assay listed above. PCR products were purified using ExoSAP-IT reagent (USB, Cleveland, OH) following the manufacturer's protocol and sequenced with Cs1482F using a BigDye Terminator (version 3.1) cycle sequencing kit on an ABI Prism 3730 genetic analyzer at the Center for Genome Research and Biocomputing, OSU. Data were collected via the ABI Prism 3730 data collection software (version 3.0) and processed using ABI Prism DNA sequencing analysis software (version 5.2). Parasite genotypes were assessed using BioEdit software (14), and genotype proportions were estimated using peak height averages (2). The three assays were compared with a subset of samples.

Genotype density (number of spores liter^{-1}) was compared with percent *C. shasta*-induced mortality and degree-days to death for Chinook and coho salmon and rainbow trout. April, May, and June data were used in the analyses since *C. shasta* myxospores, which are not infective to the fish host but detectable by qPCR, may be present in September and October and confound the data sets. We assigned genotype-specific lethality thresholds for Chinook and coho salmon based on a management goal to reduce mortality below 40%.

Data analyses. (i) Parasite density. We used a three-way analysis of variance (ANOVA) to test for differences in parasite density among sites, months, and years (spotfire S+ software, version 8.1; TIBCO, Palo Alto, CA). Cq values were log transformed to meet normality assumptions. If significant differences were detected, multiple comparisons were conducted using Tukey's crosswise comparisons. October 2009 data were

excluded from the analyses (this was a single event that did not overlap other survey years, and no mortality was observed).

(ii) Model development and validation. We constructed two logistic regression models to evaluate the predictive capabilities of either total density (total density model) or total genotype-specific density (genotype model) of *C. shasta* for parasite-induced mortality of Chinook and coho salmon in the KR. The baseline models also consisted of environmental factors, and the final variables selected are listed in Table S3 in the supplemental material. The models were created using 80% of the observed data, with the remaining 20% held in reserve to evaluate their predictive capabilities. The observed mortality for each species was rounded up to the nearest integer, and a quasi-Poisson distribution was used. Proportions of genotypes present were not obtainable for all time points; hence, we used the average of available data points for each site to fill gaps. Logs of both total parasite density and genotype-specific density were used to develop their respective models. The other explanatory variables were species, exposure site, temperature during exposure, rearing temperature, and interaction terms between dose and all the other terms and between species and exposure site. We conducted an analysis of deviance to determine the relative importance of each parameter because Akaike information criterion values were not produced for a quasi-Poisson distribution. Nonsignificant parameters were removed individually, and the model was reanalyzed until only statistically or biologically significant parameters remained. A goodness of fit for the final model was approximated by 1 − (residual deviance/null deviance) for each model. We estimated the odds ratio, or risk, of either site or species parameters, relative to BC and Chinook values at 13°C, by taking the inverse of the exponent of the parameter coefficient of interest (site, species, or rearing temperature). To determine the predictive accuracy of the final model, data from the validation data set were entered and the predicted value was compared to the observed data. We then plotted the predicted mortality values for each model versus the observed mortality and estimated a linear regression and R^2 value. Lastly, the Brier score (average prediction error) was estimated for each final model as described previously (27). Model development, parameter estimation, and validation were performed using R statistical software (26).

A subset of sentinel fish and water sample data (BC, May and June 2007 to 2010) are used by Ray et al. (25) in their temperature/mortality analysis.

RESULTS

Sentinel fish exposures. The impact of *C. shasta* on salmonids exposed in the KR varied among sites, among years, and between fish species and was affected by holding temperature (Fig. 2; see Table S2 in the supplemental material). The positive-control fish, nonindigenous rainbow trout, were most susceptible to *C. shasta* and had high mortality from May to September at most sites. For example, in 2010, >90% of rainbow trout died following exposure in June at OR, SV, and BC (mortality at KB was <5%) (data not shown). At the same time points, native Chinook and coho salmon had their lowest losses of the 5-year study (≤20%) (Fig. 2). The highest losses of Chinook occurred following exposure at BC in 4/5 years; in 2008 and 2009, 87 to 89% of fish held at 15°C died (Fig. 2). Losses of both Chinook and coho were generally highest in May and June (see Table S2 in the supplemental material). From 2007 to 2009 there was a shift in the level of *C. shasta*-induced mortality of Chinook compared with coho (Fig. 2). At BC in June 2007, juvenile coho loss (82%) was higher than juvenile Chinook loss (40%). In 2008, both species had high losses (85 to 89%). In 2009 the situation reversed, with the Chinook loss (87%) being higher than the coho loss (58%). There was a clear temperature effect on percent parasite-induced mortality in both Chi-
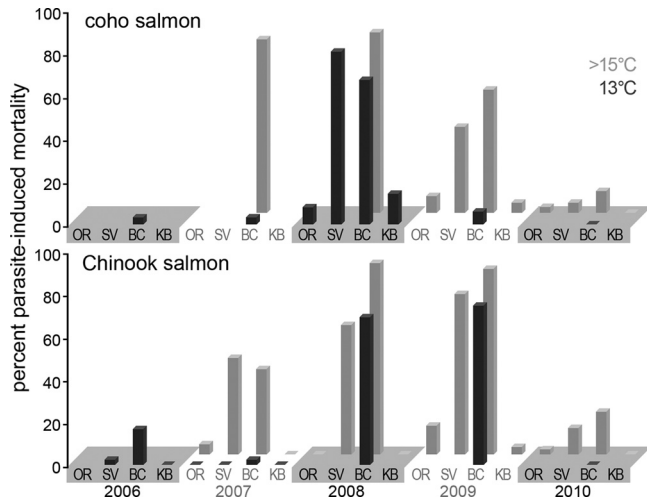
FIG 2 *Ceratomyxa shasta*-induced mortality of coho and Chinook salmon after a 3-day exposure at four lower Klamath River main stem index sites in June 2006 to 2010. Fish were held at ambient (13°C) and elevated (>15°C) temperatures in the laboratory. Sites are ordered in direction of flow (right to left represents east to west); empty cells indicate that no fish were held at that time/site/temperature.

nook and coho held at two different temperatures postexposure; more fish died at >15°C than at 13°C (Fig. 2).

Water samples. (i) Protocol changes. Filter membrane dissolution in acetone prior to DNA extraction reduced sample handling time compared with that for cutting up of the filter discs but increased the overall processing time. Acetone-processed samples fluoresced sooner in the qPCR than cut samples: river water samples fluoresced on average 1.7 cycles earlier, lab culture water 1.7 cycles earlier, myxospores 1.7 cycles earlier, and actinospores 1.2 cycles earlier. Cq standard deviations of replicate samples on the qPCR plate were lower for the acetone-processed samples and reflected reduced inter- and intrauser variation.

The greatest variation among replicate field water samples occurred with samples with ≤1 spore liter^{-1}; there was less variation among samples containing >1 spore liter^{-1}. Variation among replicates collected by the automatic sampler (technical replicates) was less than that among samples collected manually (biological replicates).

(ii) Parasite densities. *Ceratomyxa shasta* was detected in water at all four index sites, but parasite density differed significantly by one-way ANOVA between sites ($F_3 = 10.91$, $P = 0.0001$), months ($F_3 = 4.61$, $P = 0.0010$), and years ($F_4 = 7.87$, $P = 0.0002$) (Fig. 3). Parasite densities varied geographically, seasonally, and annually, but at each site, seasonal density patterns were more consistent. No difference between months or years within site was detected. The fewest spores were present at the uppermost site (KB; overall average Cq = 36.1), the most spores were present at the neighboring downstream site (BC; Cq = 32.1), similar levels were present at the next site (SV; Cq = 32.4), and then densities decreased downstream (OR; Cq = 36.0) (Fig. 3; note that samples with more target DNA fluoresce sooner in the qPCR and have lower qPCR values). On average, parasite densities were highest at the two neighboring sites, BC and SV, across all months and years and differed significantly from those at KB. The general temporal pattern among years was that waterborne parasites increased from
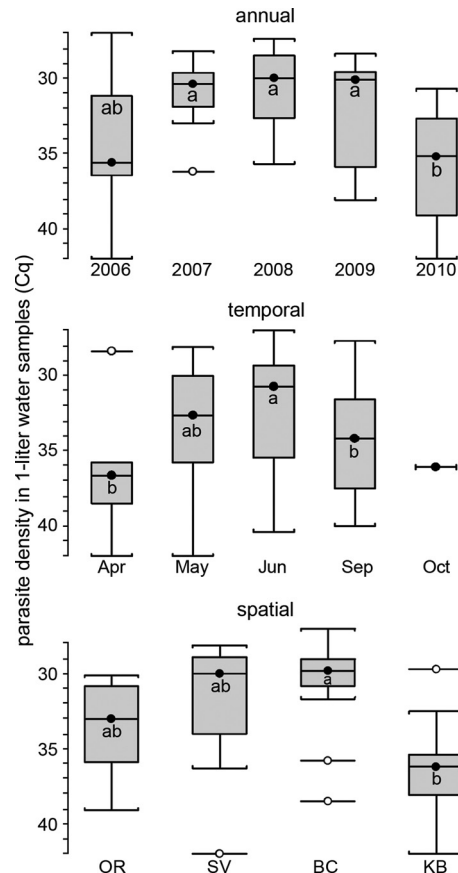

FIG 3 Density of *Ceratomyxa shasta* in 1-liter water samples collected during a sentinel fish exposure (three 1-liter samples collected at the start and end) at four Klamath River main stem index sites from April through October 2006 to 2010. Annual density data are the average density of 1-liter samples from all years and all sites in April, May, and June. Temporal density is the average density of 1-liter samples from all four index sites in all months in all years. Spatial density is the average density of 1-liter samples from all sites in April, May, and June in all years. A Cq of 34.5 represents ~1 spore liter^{-1}. Filled circles denote median values. Data outside the 95% confidence intervals are indicated by open circles. Groups with different letters (such as "a" or "b") are significantly different.

April (Cq = 36.3) to May (Cq = 33.1), peaked in June (Cq = 32.0), and then decreased in September (Cq = 34.5) (Fig. 3). The exception was in 2009, when densities peaked early, in April, and then declined. Densities in April and September were significantly lower than in June. Average annual parasite density was greatest in 2008 (Cq = 30.7) but was not significantly different between 2008 and 2007 (Cq = 31.0), 2009 (Cq = 32.2), or 2006 (Cq = 34.4). The greater variation observed in 2006 was most likely because of low parasite densities at SV. Average density was significantly lower in 2010 (Cq = 36.0) than in the preceding 3 years.

(iii) Genotype. There were no differences in *C. shasta* ITS1 genotype composition or proportion among the three genotyping assays. The nested assay was most successful in amplifying poor-quality or low-quantity DNA. More samples than we were able to sequence were qPCR positive. In particular, there was often insufficient parasite to sequence in September and October samples. Water samples were more difficult to sequence than fish tissue samples; from 2006 to 2010 we sequenced, respectively, 6/7, 24/31, 21/29, 45/69, and 12/44 water samples.
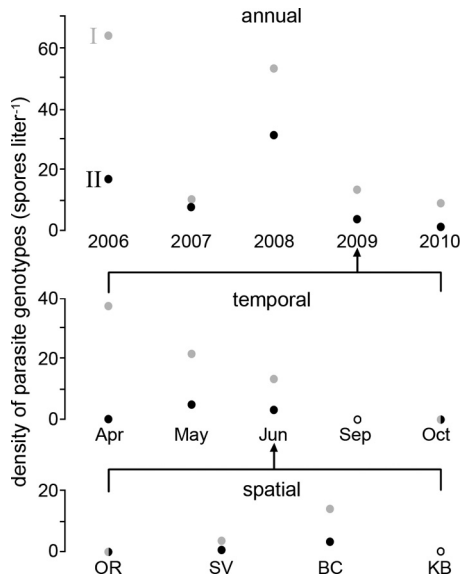
FIG 4 Genotype-specific density (number of spores liter^{-1}) of waterborne *Ceratomyxa shasta* in the lower Klamath River main stem. Annual density is shown for an index site above the confluence with BC in June from 2006 through 2010. Temporal density is shown for BC from April through October from 2006 to 2010. Spatial density is shown for all four main stem index sites in June 2009. An open circle denotes a sample that was unsequencable.

Interannual trends in parasite genotypes were determined for BC only from June 2006 to 2010. In all years, genotype I was as prevalent or more prevalent than genotype II (Fig. 4). The seasonal trend in genotypes was determined from BC samples from April to October 2009. Spatial trends among the four sites were compared in June 2009. Genotype I dominated all samples (Fig. 4). The shift in level of *C. shasta*-induced mortality of Chinook compared to coho from 2007 to 2009 corresponded to a shift in genotype-specific spore density: genotype II decreased in relative abundance (7.8 to 3.4 spores liter^{-1}), whereas genotype I increased in relative abundance (9.7 to 13.6 spores liter^{-1}); total spore density remained similar between years (16.9 to 19.0 spores liter^{-1}) (Fig. 5).

Mortality was genotype specific and density dependent (sigmoidal) for both Chinook and coho salmon (Fig. 6). At >15°C, 10 spores liter^{-1} genotype I resulted in ≥40% mortality in Chinook, whereas <5 spores liter^{-1} genotype II caused high mortality in coho (Fig. 6). In Chinook, duration of disease correlated with
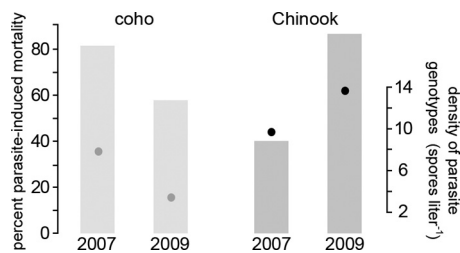

FIG 5 Correlation of *Ceratomyxa shasta*-induced mortality of coho and Chinook salmon and genotype-specific density of waterborne parasite in 2007 and 2009. Although total parasite density was similar between years, coho mortality decreased, as did the relative abundance of genotype II. The converse was observed for Chinook and genotype I.
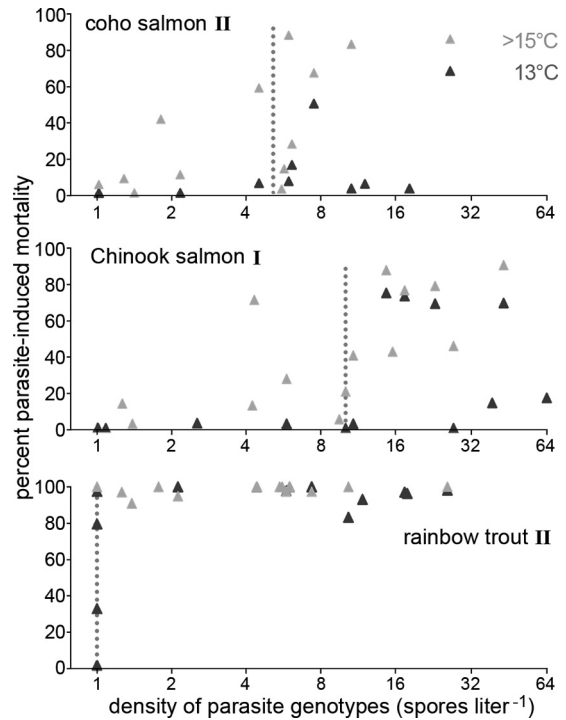

FIG 6 Relationship between *Ceratomyxa shasta*-induced mortality of coho and Chinook salmon and nonindigenous rainbow trout and density of genotype-specific waterborne parasite in the lower Klamath River (four index sites) from 2006 to 2010 (April, May, June). Each data point is for a group of 25 to 60 fish (see Table S1 in the supplemental material for specifics). The density threshold for 40% mortality is marked with a dotted line; this represents a management goal.

temperature rather than spore density: death took 427.3 ± 8.5 degree-days (dd) at 13°C and 357.2 ± 5.8 dd at >15°C (Fig. 7). In contrast, disease progression in coho positively correlated with spore density at both temperatures; however, density explained only 8 to 9% of the variation (Fig. 7): death took 546.6 ± 21.0 dd at 13°C and 562.6 ± 5.8 dd at >15°C. Nonindigenous rainbow trout incurred high mortality at low parasite levels (1 spore liter^{-1} caused 100% mortality), and similar to Chinook, disease progression was independent of spore density but was influenced by temperature (Fig. 7): on average, mortality occurred in 507.4 ± 5.6 dd at 13°C and in 396.1 ± 3.4 dd at >15°C.

Predictive model development and validation. Neither logistic regression model (total density or genotype-specific density) identified host fish species (Chinook or coho) as a statistically significant parameter (see Table S3 in the supplemental material), but we retained this parameter for biological interest. Both models identified log$_{10}$ parasite density, site, and rearing temperature to be significant; however, the total density model identified a significant interaction term between log$_{10}$ density and rearing temperature. Both models indicated that site was strongly associated with mortality, and we determined the odds ratio, or risk of mortality, at each site, compared with BC: for example, in the total density model, the odds of a Chinook salmon dying at BC were ~8 times higher than that at KB. Although the trends held true in the genotype model, the magnitude of the odds was lower (~6). In the total density model, the 95% confidence intervals (CIs) around the means of both the intercept and the species parameter overlap 0,
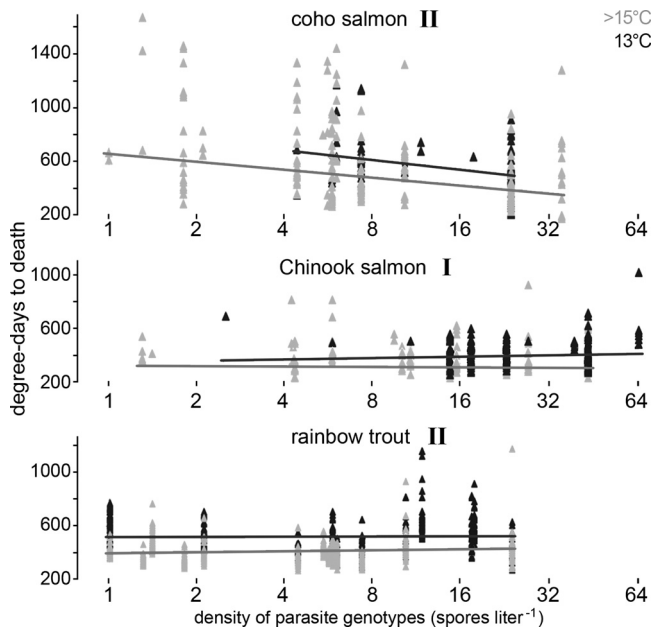
FIG 7 Relationship between *Ceratomyxa shasta*-induced degree-days to death for coho salmon, Chinook salmon, and out-of-basin rainbow trout and genotype-specific density of waterborne parasites in the lower Klamath River (four index sites) from 2006 to 2010 (April, May, June). Each data point is an individual fish.

which suggests highly variable effects of these parameters on the predicted mortality. In the genotype model, the 95% CIs of the species parameter also overlap 0, which again indicates unpredictable effects on predicting mortality. Both models had almost equal approximated R^2 values (~0.6); however, the genotype model used one less parameter, which suggests that this is a slightly better model for predicting mortality. Neither model was accurate in its predictive capability, but the genotype model predictions fitted the observed data ($R^2 = 0.29$) better than the total density model predictions ($R^2 = 0.16$) (see Figure S4 in the supplemental material). The genotype model also had smaller confidence intervals around the predicted mortality points, with 2/19 upper limits of >100% mortality, whereas the total density model had 3/19 upper bounds of <100%. Another measure of the models' predictive abilities is the Brier score, where 0 equals a perfect fit and 0.25 is an unusable model. The Brier score for both models was near 0; the genotype model scored 0.04, slightly better than the total density model with 0.06.

DISCUSSION

Approach and methodology. Monitoring of *C. shasta* in the KR has primarily involved exposure of caged sentinel fish (19, 28) and sampling of free-ranging fish (30). Use of native fish species, principally, Chinook and coho salmon, provides the most accurate reflection of wild populations but involves lethal sampling of sensitive species. Readily available, nonindigenous rainbow trout have been used in sentinel studies (19, 28), but we discovered that they were an unsuitable proxy for native salmonid species: although trout were more susceptible to the parasite, they became infected with only a subset of parasite genotypes (2, 3).

An alternative parasite-monitoring strategy is to directly sample river water, which does not require fish and enables more frequent data collection and faster data generation. However, quantification of waterborne *C. shasta* gives a measure of instantaneous parasite density in the river rather than the actual infectious dose that fish receive, which is a product of density (genotype specific, number of spores liter^{-1}), water flow (liters min^{-1}), and length of exposure (min). Lethal infectious doses are estimated to be 1 spore for rainbow trout (7, 8) and ~75,000 spores for KR fall Chinook (24); the lethal dose for coho is unknown. Because water sampling analyzes a fraction of the volume that fish are exposed to, the pathogen may be missed at low spore densities (<1 spore per 3 liters), at which fish may still become infected. Use of a programmable automatic sampler provided a better representation of exposure dose than manual sampling, since water collection could be more frequent and cover a more extensive sampling period than is practical manually.

Estimates of exposure dose of *C. shasta* spores are further complicated by the existence of multiple, host-specific genotypes of the parasite. We found that fluctuations in genotype proportions among years paralleled the mortality levels of their respective hosts (genotype I, Chinook; genotype II, coho). For example, a switch in the level of mortality of Chinook and coho salmon exposed near BC from 2007 to 2009 could not be explained by total parasite density, which was similar between years (25; this study), but was explained by changes in density of specific genotypes. Indeed, we found that host-specific parasite density explained 65% of the variation in mortality observed in Chinook and coho salmon, and these data support the use of direct parasite quantification from water samples as a management tool.

Biological effects on salmon. Disease severity is a function of parasite dose and virulence, the inherent resistance of the fish strain, and temperature (18). Mortality and time to death are two measures of disease severity. Generally, a host is considered more susceptible than another if fewer parasites cause a similar level of mortality or if it dies sooner from infection with the same dose. We discovered that each KR salmonid species responded differently to its respective parasite genotype: generally, Chinook required twice as many spores as coho to reach the same percent mortality (see also reference 25). However, Chinook died more rapidly than coho at lower spore densities. Time to death was relatively constant for Chinook, irrespective of spore density, whereas disease progressed more quickly in coho as parasite levels increased. These observed fish-specific combinations of host responses are inconsistent with our general definition of susceptibility.

We hypothesize that the differences in host-parasite dynamics of Chinook and coho salmon that we observed reflect differences in their life histories. Parasite development may progress faster in Chinook because juveniles may leave the river system for the ocean within several weeks; hence, mature parasite spores must be produced within this time frame if they have any chance of encountering the obligate freshwater polychaete host. In contrast, juvenile coho may reside in freshwater for a year, and hence, there is no selection pressure for the parasite to complete spore development as rapidly. Rearing location may also influence parasite exposure: Chinook may tolerate higher parasite levels than coho because they spend most of their time in the river main stem, where the majority of actinospore production occurs, whereas coho tend to inhabit tributaries primarily, which contribute negligible numbers of parasites, and thus, their normal exposure is lower (parasite densities are our unpublished data). Our time-to-

death data represent individual fish and provide an indicator of intraspecific variation. Disease progression was relatively uniform among Chinook, whereas there was considerable variation among the coho, which suggested that the Chinook were more genetically homogeneous.

Linkage to population-level effects. Although our sentinel fish received a shorter exposure (3 days) to *C. shasta* than free-ranging KR salmonids, the mortality trends of the sentinel fish paralleled those of outmigrants. The U.S. Fish and Wildlife Service California-Nevada Fish Health Center monitors the weekly incidence of *C. shasta* infection in juvenile outmigrating KR salmon and uses histological assessment of salmon captured above the Trinity River confluence for comparing disease prevalence year to year. From May to July 2006 to 2010, infection was highest in 2009 (54%) and lowest in 2010 (15%) (30). In 2006, the temporal shift and lower magnitude of *C. shasta* infection in outmigrant fish were associated with high precipitation and a large flow event in the spring. However, prevalence of infection decreased only temporarily, and the 2006 annual prevalence of infection was not substantially lower than that in 2007, which had no significant flow events (30). In 2010, prevalence of infection, mortality, and spore density were relatively low. River flows below Iron Gate Dam were not substantially different from those in other years but spring/early summer temperatures were cooler (1.5 to 2°C), and, hence, temperature appears to have played a greater role in disease dynamics that year (25, 30).

River temperatures and flows are interconnected factors that influence disease in salmonids. Temperature affects all aspects of the host-parasite cycle, including parasite development in both hosts and longevity of waterborne stages (23, 31). Elevated water temperatures have been linked to increased respiration rates in salmon (9), potentially increasing the number of infectious stages that pass over the fish's gills and thus the probability of infection. Decreased flows have been associated with increased *C. shasta* infection (7), which has also been observed for another myxozoan parasite, *Myxobolus cerebralis* (16). In contrast, higher flows may dilute infectious stages and affect transmission efficiency. The survival rate of hatchery-released juvenile fall Chinook that migrated through a *C. shasta*-infectious region of the KR main stem, between the Shasta River (located between our KB and BC sites) and Seiad Creek confluence (our SV site), correlated significantly with stream discharge in this reach (13). Differences in flow and temperature may explain the strong intersite differences that we observed for *C. shasta*, and our future studies will incorporate flow measurements. Abiotic and biotic factors are intrinsically intertwined, and understanding their relative importance in disease dynamics may highlight measures for ameliorating the current imbalance in the KR.

While ceratomyxosis clearly has a population-level impact, the sublethal effects of this parasite are less understood. Infection may delay smoltification once juveniles reach the estuary, retard growth, impair swimming performance, affect body condition, and increase stress levels and susceptibility to secondary infections.

Management. Although the density of waterborne stages of *C. shasta* varied annually, there were consistent patterns in the spatial and temporal distribution of the parasite in the lower KR. The parasite was most prevalent in early summer at all index sites, and two sites consistently had the highest spore levels. The source of these parasite stages and the extent of this infectious zone within the 212-Rkm study region remain unknown and warrant additional, intensive sampling to focus management efforts.

As the majority of Chinook and coho in the lower KR are of hatchery origin, there is the potential to alter the time of their release from the hatchery into the river to avoid peak actinospore densities or other unfavorable river conditions. Although this approach was recommended over 2 decades ago (19), it has not been implemented. Neither the size nor the age of a fish appears to be related to infection (7); thus, fish development at time of release will not likely affect the infection outcome. Other management options that are being explored include altering the flow schedule from Iron Gate Dam and introducing pulse flows to destabilize polychaete habitat. However, it is difficult to meet the needs of all interested parties in the upper and lower KR: the farmers, fishers, Native American tribes, and the different fish species.

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