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Mortality threshold for juvenile Chinook salmon *Oncorhynchus tshawytscha* in an epidemiological model of *Ceratomyxa shasta*

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ABSTRACT: The myxozoan parasite *Ceratomyxa shasta* is a significant pathogen of juvenile Chinook salmon *Oncorhynchus tshawytscha* in the Klamath River, California, USA. This parasite requires 2 hosts to complete its life cycle: a freshwater polychaete (*Manayunkia speciosa*) and a salmonid. The complex life cycle and large geographic area where infection occurs make it difficult to monitor and manage ceratomyxosis. We present a model for ceratomyxosis-induced mortality in *O. tshawytscha*, from which parameters important to the persistence of *C. shasta* are identified. We also experimentally quantify specific parameters from the model and identify a mortality threshold (a critical parameter), by naturally exposing native *O. tshawytscha* to *C. shasta* in the Klamath River. The average percent mortality that resulted from these experimental challenges ranged from 2.5 to 98.5% over an exposure dose of 4.4 to 612×10^6 parasites. This experiment identified a non-linear mortality threshold of $7.7 \pm 2.1 \times 10^4$ actinospores fish^{-1} for Chinook salmon from the Iron Gate Hatchery on the Klamath River. Below this threshold no mortality occurred and above it mortality increased dramatically, thus providing a target by which to reduce parasitism in emigrating juvenile *O. tshawytscha*.

KEY WORDS: *Ceratomyxa shasta* · Epidemiological model · Mortality threshold · Chinook salmon · Disease ecology · Myxozoan · Macroparasite

INTRODUCTION

Declining populations of Chinook salmon *Oncorhynchus tshawytscha* in the American Northwest are a major concern ecologically, economically, and sociologically. Management of this species is complicated due to the numerous biological and environmental interactions encountered at each life stage, including loss from disease. In the Klamath River, California, USA, the myxozoan parasite, *Ceratomyxa shasta,* is a significant pathogen of juvenile *O. tshawytscha* (Foott et al. 1999, 2004), with infection prevalence in outmigrant populations ranging from 30 to 60% since monitoring began in 1994 (Nichols & True 2007, Nichols et al. 2008). *C. shasta* has 2 distinct environmental stages and requires 2 specific hosts to complete its life cycle (see Fig. 1). Initially described in 1950 from an outbreak in a rainbow trout hatchery (Noble 1950), it was not until 1997 that the freshwater polychaete *Manayunkia speciosa* was identified as the invertebrate host (Bartholomew et al. 1997). Since there is no known treatment for ceratomyxosis it is essential to identify critical points in the parasite life cycle where management strategies can be applied, thereby decreasing morbidity and mortality in out-migrating juvenile fish.

Various epidemiological models have been used to explain the complexities of host–parasite interactions and to identify the parameters necessary for the perpetuation of disease. Using these parameters one can create a system of equations and calculate the 'basic reproduction number' (R_0), defined as the number of secondary infections arising from a primary case (Dietz 1993, Smith et al. 2007). R_o has an intrinsic threshold

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value of 1, and below this threshold a pathogen cannot maintain itself within the population; however, when $R_o > 1$ the pathogen will spread throughout a naïve population (Macdonald 1952, Reno 1998). Thus, identifying R_o for a particular pathogen provides information on how severe a disease outbreak is likely to be and what kind of management effort may be required to reduce it. These models have been successfully developed for infectious diseases of humans, the first being the population dynamic model of malaria developed by Ross (Ross 1911, Smith et al. 2007). Epidemiological models have been applied to diseases occurring in wildlife populations; however, they tend to be biased towards large-bodied and easily observable hosts (Dobson & Foufopoulos 2001). One model commonly used for examining the effect of pathogens on cultured fish has been the Susceptible-Infected-Recovered (SIR) model. Although this model works well for directly transmitted pathogens such as infectious haematopoietic necrosis virus (IHNV) and the bacterium *Aeromonas salmonicida* (Ögüt 2003), for aquatic pathogens that require multiple hosts their application becomes increasingly complex, involving multiplicative SIR models. Thus, SIR models are useful only if the appropriate information on the various components is available (Reno 1998).

In this paper we introduce a mathematical model for the *Ceratomyxa shasta* life cycle, adapted from the Ross (1911) malaria population dynamic model, to identify parameters that need quantification (Table 1). We then experimentally quantified actinospore dose (A) and the parasite-induced mortality (δ) from that dose. From these data we calculated a mortality threshold for Chinook salmon from the Iron Gate Hatchery (IGH) on the Klamath River.

MATERIALS AND METHODS

Model development. Our model, presented in Fig. 1 and Table 1, and its system of equations were modified from a model describing the life cycle of a 'digenean-type' parasite with 2 free living stages (Dobson 1988). The number of actinospores and the parasite related mortality are directly measured in the experiments described below. Indirect measurements for the transmission rate (η_1) and prevalence of infection in the Chinook salmon host (C) were estimated from the field exposures below.

Fish and study area. Juvenile (first year age class, 0+) fall *Oncorhynchus tshawytscha* were obtained from the California Department of Fish and Game, IGH and were transported to the study site in aerated coolers. Fish were naturally exposed to *Ceratomyxa shasta* in the main stem of the Klamath River, about 1 river kilometer (Rkm) upriver from the confluence with Beaver Creek (259.1 Rkm, measured from the mouth of the river at the Pacific Ocean). The study site is about 45 Rkm downriver from Iron Gate Dam and within a reach of the river where high densities of *C. shasta* have been measured (Stocking & Bartholomew 2007). In order to increase the range of parasite

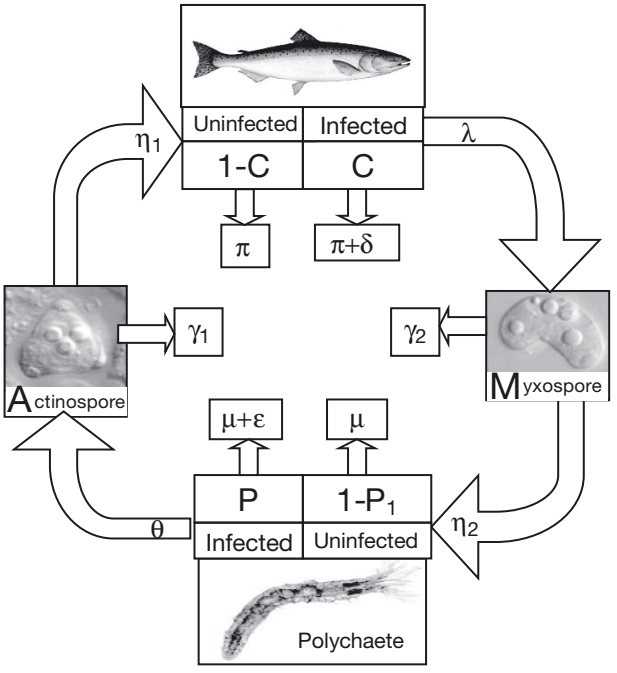

Fig. 1. Flow chart for the life cycle of *Ceratomyxa shasta* and its hosts. The myxospore stage (M) infects the polychaete host (P), which produces the actinospore stage (A) that infects the salmonid host (S). The experiments in this study provide a series of values for the transmission (η_1) of actinospores to the Chinook salmon host (C) and the resulting mortality (δ). For further definitions of parameters see Table 1. The differential equations (Eqs. 1 to 5) are detailed in 'Results: Model development'

Table 1. Parameters used in the epidemiological model for *Ceratomyxa shasta*

Symbol	Parameter
P	Proportion of infected polychaete
C	Proportion of infected Chinook salmon
A	Number of actinospores
M	Number of myxospores
η_1	Transmission rate to salmon
η_2	Transmission rate to polychaete
λ	Myxospore production
θ	Actinospore production
π	Natural mortality of salmon
δ	Parasite-induced mortality of salmon
μ	Natural mortality of polychaete
ε	Parasite-induced mortality of polychaete
γ_1	Loss of actinospores
γ_2	Loss of myxospores

doses, 2 separate exposures were conducted, one in June and then again in September 2008. Fish were handled similarly before and after each exposure. Twenty unexposed IGH Chinook salmon were held as controls to ensure no background infection was present. Controls were processed in an identical manner to the exposed fish.

Fish exposures. IGH Chinook salmon were exposed in the Klamath River in eight 0.28 × 1 m cylindrical PVC cages, secured to the river bed with rebar braces on both sides. Screening (0.64 cm mesh) on each end of the cage allowed for a natural flow. In June, 40 *Oncorhynchus tshawytscha* were each placed into Cages 1 to 4 for 72 h to determine the variation of parasite dose between cages. To assess dose effects, 20 fish were added to Cages 5 to 8 every 24 h for 3 d, for a total of 60 fish cage^{-1}. To obtain low-end estimates of parasite dose and dose effects, this experiment was repeated, with some modifications, in September when a lower parasite challenge was expected. In September, 12 to 15 fish were added to each of the 8 cages at 72, 48, 24, and 16 h, for a total of 60 fish cage^{-1}. In June and September, each exposure group was assigned a unique fin clip to allow for separation of the groups at the end of the challenge. After exposure, each group of fish was transferred to a separate aerated cooler, transported to the Oregon State University Salmon Disease Lab and relocated to 25 l tanks with 18°C specific pathogen-free water for the duration of the study (~90 d post exposure, dpe). In the June study, salmon from Cages 5 to 8 were inadvertently combined into a single aquarium for each exposure period. Consequently, variation between cages cannot be directly measured. Preventative treatments for bacterial infections and external parasites were administered as described by Stocking et al. (2006). Fish were fed and observed twice daily. Sick and moribund fish were removed, euthanized with an overdose of MS-222 and either immediately examined for infection or frozen for future examination. Fish surviving 90 dpe were sacrificed and immediately examined for infection.

Actinospore dose. To determine the actinospore dose, the average daily water velocity during the exposure was multiplied by the average daily density of the parasite. Water velocity through each cage was measured with a Global Water Flow Probe (Global Waters) every 2 h for the first 24 h and then every 4 h for the remaining 48 h. These measurements were averaged for each 24 h period and multiplied by the volume of the cage to determine the average daily flow. To determine the parasite density 1 l of water was collected every 2 h by an automated water sampler (Teledyne Isco) and pooled in a 15 l container. After 24 h of collection, four 1 l sub-samples were collected from the container. All samples were individually filtered and 3 of the 4 samples were processed for quantification of parasite DNA by quantitative polymerase chain reaction (qPCR) as described by Hallett & Bartholomew (2006). Each sample was analyzed in duplicate on a single 96-well qPCR plate. The individual cycle threshold values from each of the 3 samples were averaged and used to estimate the number of actinospores per l of river water for a 24 h period. This estimate was calculated by extrapolating from a standard curve based on the value of a known number of parasites, similar to the one developed by Hallett & Bartholomew (2006). The transmission rate of the actinospore to the salmonid host was estimated by dividing the actinospore estimate by the total number of fish infected cage^{-1}.

Determination of infection. All dead and moribund fish and 5 randomly selected terminated fish from each exposure group were microscopically examined for the myxospore stage of *Ceratomyxa shasta*. Material from the posterior intestine was collected with a sterilized inoculating loop, placed on a microscope slide and examined at 200× magnification for up to 3 min (Bartholomew 2002). If no spores were observed, approximately 5 mm of intestinal tissue was removed and frozen for PCR analysis as described by Palenzuela et al. (1999). The prevalence of infection in the Chinook salmon host was estimated by summing the observed ceratomyxosis-related mortalities and the fish that were found positive for infection by PCR.

Data analysis. Chinook salmon that died after 5 dpe were included in the analysis; earlier mortalities were ascribed to non-*Ceratomyxa shasta* causes. Percent mortality was calculated by combining fish that were positive for *C. shasta* either by microscopy or PCR analysis and dividing the number of positive fish by the total number exposed in each cage for each exposure period. The percent of infection mortality for each cage in an exposure period was determined for the entire observational period (90 dpe). Statistical analysis was conducted using S-plus 8.0 (TIBCO software). Kaplan-Meier survival curves were analyzed by Cox proportional hazards test, using score (logrank) tests to determine the difference in mortalities between cages and exposure periods. One sample, pair wise *t*-tests and one-way ANOVAs were used to determine the variations in flow between days and between cages.

RESULTS

Model development

The structure and parameters of this model (Fig. 1 and Table 1, respectively) are represented by a flow diagram based on the life cycle of *Ceratomyxa shasta*. A series of 4 differential equations was developed that

describes the transmission and mortality rates at each stage in the life cycle. For the development of these equations we assume *C. shasta* is a microparasite. For definitions of all parameters used in the equations see Table 1.

Salmon infection and mortality. Transmission of actinospores to uninfected salmonid host, less the natural and parasite-induced mortality of the host.

$$\frac{dC}{dt} = \eta_1 A(1-C) - \pi(1-C) - (\pi+\delta)C \quad (1)$$

Myxospore transmission and mortality. Production of myxospore from infected salmon, less those spores transmitted to uninfected polychaetes and spores that are lost to the environment.

$$\frac{dM}{dt} = \lambda C - \gamma_2 M - \eta_2 M(1-P) \quad (2)$$

Polychaete infection and mortality. Transmission of myxospores to uninfected polychaetes, less the natural and parasite induced mortality of the polychaete host.

$$\frac{dP}{dt} = \eta_2 M(1-P) - \mu(1-P) - (\mu+\varepsilon)P \quad (3)$$

Actinospore transmission and mortality. Production of actinospores from infected polychaete, less spores transmitted to uninfected salmon and spores that are lost to the environment.

$$\frac{dA}{dt} = \theta P - \gamma_1 A - \eta_1 A(1-C) \quad (4)$$

Eqs. (1) to (4) can be solved for the equation for R_o (Eq. 5). This equation describes the transmission of myxospore to polychaete (T_{MP}) and actinospore to salmonid (T_{AC}) divided by the mortalities for both spore stages (M_M and M_A) and hosts (M_P and M_C). The experiments conducted in this study provided a range of measurements for the parameters in Eq. (1): actinospore dose, the resulting parasite-induced mortality and indirect measurements of the transmission of the actinospore to the salmonid and the proportion of salmonid population infected.

$$R_0 = \frac{\lambda\eta_1(1-P)\ \theta\eta_2\ (1-C)}{[\mu+(\mu+\varepsilon)][\pi+(\pi+\delta)][\eta_1(1-C)+\gamma_1][\eta_2(1-P)+\gamma_2]} = \frac{T_{MP}T_{AC}}{M_P M_C M_M M_A} \quad (5)$$

Fish infection

We evaluated parasite-induced mortality following different exposure durations and expect these variables to be linearly proportionate. Average percent mortality for the June 72, 48 and 24 h exposure groups was 94.2, 98.5 and 84.7 %, respectively. The average percent mortality of fish in Cages 1 to 4, exposed for 72 h, was 95.1 ± 2 %, and did not differ significantly between these 4 groups (score (logrank) df = 3, p = 0.504). The average percent mortality of the pooled fish exposed for 72 h (90.6 %, Cages 5 to 8) was not statistically different from that observed in Cages 1 to 4 (score (logrank) df = 4, p = 0.465), indicating similarly minimal variability in mortality between Cages 5 to 8. Fish from Cages 5 to 8 were inadvertently combined into aquaria for each holding group, thereby prohibiting the calculation of variance in parasite-induced mortality. However, given the high actinospore dose and the limited variability between the 72 h exposure in June, it can be assumed that there was minimal variability between these cages and thus these exposures were not repeated. Although the difference in mortality between all 3 exposure periods was statistically significant (score (logrank) df = 2, p < 0.001) (Table 2), biologically, all 3 periods resulted in mortality too great to identify a mortality threshold.

In September, average mortality was 34.9, 17.7, 16.7 and 2.5 %, for the 72, 48, 24 and 16 h exposure periods, respectively. Differences in mortality between cages were not significantly different for the 72 and 24 h exposure periods (score (logrank) df = 7, p = 0.147 and 0.874, respectively), but a statistical difference was observed between the 48 and 16 h exposures (score (logrank) df = 7, p = 0.029 and 0.009, respectively). These differences are a result of elevated mortalities in a single cage for each exposure period. From the 48 h exposure group, 7 fish from Cage 3 died (~50 %) compared to 20 % or less for the remaining cages. In the 16 h exposure group, 3 fish (20 %) succumbed to ceratomyxosis in Cage 7 compared to no parasite-related mortality in any other cage. Percent mortality was significantly different between the

Table 2. Comparison of total *Ceratomyxa shasta* dosage and resulting mortality of Iron Gate Hatchery Chinook salmon *Oncorhynchus tshawytscha* exposed in the Klamath River, California, USA, for different durations in June and September 2008. NA: not available

Exposure duration (h)	June		September	
	Actinospore dose ($\times 10^6$)	Mortality (%)	Actinospore dose ($\times 10^6$)	Mortality (%)
16	NA	NA	4.4 (± 1.3)	2.5 (± 7.1)
24	535.4 (± 41.5)	84.7[a]	6.6 (± 2.0)	16.7 (± 6.6)
48	594.5 (± 48.2)	98.5[a]	13.2 (± 3.7)	17.7 (± 13.3)
72	612.0 (± 51.0)	94.2 (± 2.7)	153.2 (± 4.3)	34.9 (± 12.6)

[a]Pooled into 1 sample in the lab

72 and 48 h exposures (score (logrank), df = 1, $p < 0.001$) and the 24 and 16 h exposures (score (logrank) df = 1, $p = 0.001$), but not between the 48 and 24 h exposures (score (logrank) df = 1, $p = 0.759$). Mortality from ceratomyxosis was higher in June than September; however, minimal variability occurred between cages during both exposure periods (Fig. 2). All the fish that survived the duration of this study were determined to be negative for infection by the PCR assay; therefore we assume that the proportion of infected Chinook salmon is equal to the observed parasite-induced mortality. The results from these 2 exposure periods provide a range of values for our model for parasite-induced mortality, and also, in combination with the PCR assay results, an estimate of the proportion of Chinook salmon that became infected. We conclude that salmon mortality is not a constant, but is logarithmically proportional to the exposure duration.

Actinospore dose

The actinospore dose was calculated from the product of the average daily water velocities and average daily parasite density measured over the exposure duration. The average water velocity in June (5.38 ± 0.74 l s^{-1}) was significantly greater than the average velocity in September (3.93 ± 0.97 l s^{-1}, *t*-test $p < 0.05$). The average daily parasite density in June varied greatly from Day 1 to Day 3 (28, 146, 1258 spores l^{-1}, respectively). In September, the average daily parasite density was lower, yet more consistent between days (21, 20, 16 spores l^{-1}, respectively), than in June. The total actinospore dose ranged from a maximum of 612×10^6 spores, during the June 72 h exposure, to a minimum of 4.4×10^6 spores during the September 16 h exposure (Table 2). These measurements provide an array of actinospore doses that can be utilized in the model.

A lethal infectious dose $fish^{-1}$ was determined by dividing the total actinospore dose by the total number of fish $cage^{-1}$, adjusting for the increasing fish density as the experiment progressed. The individual dose ranged, approximately, from 0.05×10^6 (September, 16 h exposure) to 12.8×10^6 (June 72 h exposure) spores $fish^{-1}$, and a non-linear relationship with percent mortality can be observed (Fig. 3). We conclude that the mortality threshold in IGH *Oncorhynchus tshawytscha* was $7.7 \pm 2.1 \times 10^4$ actinospores $fish^{-1}$.

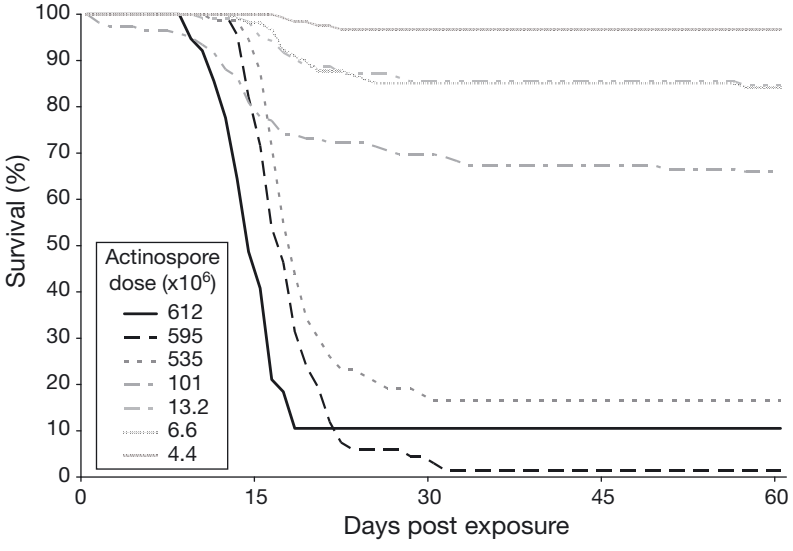

Fig. 2. *Ceratomyxa shasta* infecting *Oncorhynchus tshawytscha*. Survival curves of Iron Gate Hatchery Chinook salmon exposed to various doses of *C. shasta* in the Klamath River, California, USA, in June and September 2008. Curves are identified by the average actinospore dose received, in millions of actinospores

DISCUSSION

We developed an epidemiological model for the *Ceratomyxa shasta* life cycle as a tool for evaluating which potential management strategies might prove most effective at improving the survival of out-migrating juvenile salmonids in the Klamath River. Using this model we identified parameters and developed a system of linearizable equations necessary for transmission of this parasite. Values for many of these parameters had not been previously determined, and we chose to focus on infection in the salmonid host, utilizing sentinel fish exposures and molecular techniques to quantify this portion of the model. Thus the critical parameters investigated in the exposure experiments were: actinospore dose, proportion of salmonids infected and parasite-induced mortality (Fig. 1). From these values a mortality threshold for IGH Chinook salmon held at 18°C was determined to be $7.7 \pm 2.1 \times 10^4$ actinospores $fish^{-1}$, above which mortality increases non-linearly. Although this threshold may vary from year to year, it provides the first estimate for a *C. shasta* lethal dose for a resistant strain of fish. Identifying a threshold significantly above zero is important to disease management as it provides a target for reducing the in-river parasite burden that does not require complete eradication of the parasite.

Quantification of the dose–mortality relationship is an important interaction in disease ecology and is necessary for modeling diseases in natural populations (Crofton 1971, Anderson & May 1978). It has been qualitatively shown that fish

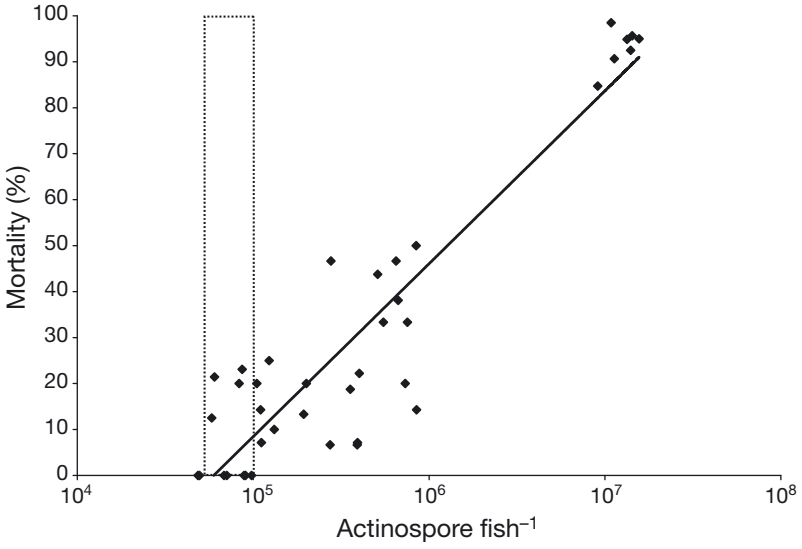

Fig. 3. Relationship between *Ceratomyxa shasta* induced mortality in Iron Gate Hatchery Chinook salmon *Oncorhynchus tshawytscha* and estimated actinospore dose fish^{-1}. The *x*-axis is log transformed to better represent the wide range of actinospore doses. The R^2 value is 0.858 and the regression equation is $y = 16.335 \times \ln(x) - 179.62$. The dotted box represents the range of values quantified for the mortality threshold

from waters where *Ceratomyxa shasta* is endemic are less susceptible to infection and mortality (reviewed by Bartholomew 1998). The first estimation of infectious dose was semi-quantitatively determined by increasing the density of fish in a given volume of water, thereby decreasing the ratio of parasites to fish. The resulting decrease in ceratomyxosis-related mortalities suggested that a single actinospore can cause mortality in a susceptible strain of rainbow trout *Oncorhynchus mykiss* (Ratliff 1983). Recent developments of laboratory challenge methods validated this prediction for susceptible rainbow trout using a quantified infectious dose (Bjork & Bartholomew 2009). In contrast, individual IGH *O. tshawytscha* exposed to 5×10^3 actinospores under identical conditions failed to become infected, indicating a higher mortality threshold for this strain (Bjork & Bartholomew 2009). To achieve an infectious dose great enough to induce mortality in these less susceptible fish, Foott et al. (2007) conducted field exposures and quantified parasite density by qPCR. In their study, Trinity River Chinook salmon were exposed to approximately 1.4×10^4 actinospores fish^{-1}, resulting in 22% mortality (Foott et al. 2007). Compared to the IGH Chinook salmon in our study, the morality threshold for the Trinity River salmon was about 5-fold lower. Although the Trinity River is a major tributary to the Klamath River, the levels of *C. shasta* are lower and the prevalence of infection in out-migrating juvenile salmon is about 1 to 3% (Nichols et al. 2008, 2009). As a result of the Trinity River salmon receiving a lower exposure in the Trinity River and having a shorter migration in the main stem of the Klamath River, this strain is likely to be more susceptible to the parasite than IGH salmon, as evident by the differences in morality thresholds. This in-basin variation in susceptibility suggests that the mortality threshold needs to be quantified for each strain in order to be used for management purposes.

The observation of a non-linear mortality threshold is crucial to understanding the epidemiology of the parasite. In general, parasites are considered either microparasites or macroparasites. For a microparasite, prevalence of infection is sufficient to evaluate parasitism in the host, and parasite dose and host mortality are not proportional. In a macroparasite relationship, parasite dose and host mortality are non-linearly related and the frequency distribution of the parasite is more important than prevalence within the host (Crofton 1971, Anderson & May 1979, May & Anderson 1979, Dobson 1988). The presence of a mortality threshold indicates a macroparasitic relationship between *Ceratomyxa shasta* and the salmonid host. Yet the ability of *C. shasta* to replicate within the salmonid host is representative of microparasitic relationships. Recognition of when *C. shasta* behaves as a macroparasite versus a microparasite within the disease cycle is critical to further the development of this model.

Application of this model is currently limited by the lack of data for various parameters. For example, estimates for the emigration rate of juvenile salmonids are highly variable, and this directly affects the potential exposure dose. A recent radio-telemetry survey documented that juvenile IGH Chinook salmon reach the estuary in a median time of 10 d (Foott et al. 2009). However, another study estimated the median travel time of coded wire-tagged IGH juvenile Chinook salmon released between 1993 and 2001 to be 32 d (Wallace 2004), over a 3-fold difference in travel time. Our study attempted to account for this difference in migration rate by using various exposure durations to reflect different exposure doses. Another limitation is that our current model does not specifically incorporate environmental or genetic variables, such as water temperature, velocity, or genetic variations within the hosts or parasites. Water temperature has been demonstrated to greatly affect mortalities from ceratomyxosis (Udey et al. 1975), yet we do not know how it affects the other parameters in the model. Similarly,

genotypic variations in *Ceratomyxa shasta* that relate to its specificity for the salmonid host have recently been identified (Atkinson & Bartholomew 2010a,b). These observations open avenues to better understanding ecological implications of environmental and genetic differences and how they can be incorporated into the dynamics of this host-parasite system.

The principal objective of this study was to develop a model and identify the parameters required for the propagation of *Ceratomyxa shasta*. The experiments in this study provided a range of values for 3 parameters, which allowed us to identify a mortality threshold for juvenile Chinook salmon. Once all the parameters are quantified, the model and resulting system of equations can be used to determine the relative sensitivity of each parameter; predict how different management strategies may influence host–parasite interactions; and, examine the resilience and reactivity of the system after management actions have been implemented (Neubert & Caswell 1997, Hosack et al. 2009).

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