Lack of Protection following Re-Exposure of Chinook Salmon to Ceratonova shasta (Myxozoa)

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Re-exposure of Chinook salmon (*Oncorhynchus tshawytscha*) to *Ceratonova shasta* *(Myxozoa)*

Running Head: *Ceratonova shasta* re-exposure

C. N. Hurst and J. L. Bartholomew*

Department of Microbiology, College of Science, Oregon State University, Campus Way, Corvallis, Oregon 97330, USA

*Corresponding author: bartholj@science.oregonstate.edu

<A>**Abstract:** The recent identification of multiple genotypes of the salmonid parasite *Ceratonova shasta* with different virulence levels in Chinook salmon (*Oncorhynchus tshawytscha*) suggests that it may be possible to immunize fish against subsequent infection and disease. We hypothesized that exposure of Chinook salmon to the less virulent parasite genotype (II) prior to the more virulent parasite genotype (I) would decrease disease and/or result in fewer mature parasites compared to fish only infected with the more virulent genotype. To test this, fish were challenged in a combination of field and laboratory exposures and we measured infection prevalence, percent morbidity, and mature parasite production. Neither mortality nor mature parasite production were reduced when fish were exposed to genotype II prior to genotype I as compared with fish exposed only to genotype I, suggesting that protection using a less virulent parasite genotype does not occur.

<A>**Introduction:** In aquaculture, there are opportunities for the control of diseases through vaccination and/or treatment. Vaccines have been developed for a variety of bacterial and viral pathogens, but no commercial parasite vaccines exist in aquaculture (Sommerset et al. 2005). However, there is evidence that fish can acquire resistance after a natural exposure to a parasite. In salmonids, resistance to parasite reinfection has been demonstrated for the microsporidians *Kabatana takedai* (Awakura 1974) and *Loma salmonae* (Speare et al. 1998; Kent et al. 1999), the monogenean *Discocotyle sagittata* (Rubio-Godoy and Tinsley 2004) and the myxozoan *Tetracapsuloides bryosalmonae* (Foott and Hedrick 1987). However, one drawback with using a re-exposure strategy based on exposure only to a virulent parasite is the inability to achieve high infection prevalence without causing a high incidence of disease and/or mortality after the initial

exposure. For example, Foott and Hedrick (1987) demonstrated that when infection prevalence with *T. bryosalmonae* was high (82%), most fish exhibited clinical disease signs.

Alternatively, prior exposure to attenuated parasites or naturally occurring less virulent parasite species/strains may also provide some protection from disease or reduce disease severity (Smith et al. 1999; Read and Taylor 2001). Attenuation decreases parasite virulence while simultaneously eliciting a protective immune response against future infections with an unaltered parasite. For example, prior exposure to an attenuated strain of the hemoflagellate *Cryptobia salmositica* protected rainbow trout (*Oncorhynchus mykiss*) against disease development (Woo and Li 1990). Protection has also been achieved for the myxozoan parasite, *Myxobolus cerebralis,* where researchers exposed fish to infective actinospores treated with UV irradiation 63 days prior to their exposure to fully infective parasites. Prior exposure resulted in decreased infection, parasite survival within the host and mature parasite production after fish were exposed for a second time (Hedrick et al. 2012). Immunization of rainbow trout to the ciliate *Tetrahymena thermophila* (a less virulent parasite species) prior to another ciliate *Ichthyopthirius multifilus* 6-10 weeks later increased host survival by approximately 50% (Wolf and Markiw 1982). Similarly, Sánchez et al. (2001) demonstrated that rainbow trout exposed to a less virulent strain of *L. salmonae* 15 weeks prior to a more virulent strain resulted in a reduction in xenoma intensity in the gills.

The recent discovery that the myxozoan *Ceratonova shasta* comprises four genotypes (0, I, II and III; Atkinson and Bartholomew 2010ab that differ in virulence presents an opportunity to immunize hatchery fish prior to their release. The life cycle of *C. shasta* is complex, with a waterborne actinospore stage attaching to and penetrating the gills of a salmonid. Once in the host, the parasite begins proliferating and travels through the blood to the intestine (Bjork and

Bartholomew 2010). The parasite then matures into the myxospore stage and is subsequently released into the water column to infect a freshwater polychaete, *Manayunkia speciosa* (Bartholomew et al. 1997). Our study focuses on Chinook salmon (*O. tshawytscha*) which are commonly infected by both *C. shasta* genotypes I and II, with genotype I causing mortality from enteronecrosis, while infection with genotype II rarely results in myxospore production or mortality (Hurst and Bartholomew 2012a). Thus, we hypothesize that infection of Chinook salmon with *C. shasta* genotype II, followed by exposure to genotype I, will result in a decrease in disease and/or myxospore production. The association of *C. shasta* with declines in adult Chinook salmon returns (Fujiwara et al. 2011) has focused attention on strategies to reduce parasite abundance and ultimately disease. Thus, immunizing hatchery fish may improve the survival of returning adults by increasing survival of out-migrating juveniles. In addition, a decrease in myxospore production could reduce the overall number of parasites in the river system by reducing transmission to the parasite's next host.

<A>Materials and Methods

Exposures.− Age 0 Chinook salmon (5-10 g) were obtained from Iron Gate Hatchery (Hornbrook, CA) and transported in aerated coolers to the John L. Fryer Salmon Disease Laboratory, Oregon State University, Corvallis, OR (SDL) and held until parasite exposure. A total of 360 fish were randomly placed into four treatments: no parasite (control-treatment 1), genotype II only (treatment 2), genotype I only (treatment 3) and genotype II, then genotype I (treatment 4). Two replicates were used for treatments 1 and 2 as no mortality was expected; treatments 3 and 4 had four replicates each (Figure 1). All replicates were comprised of 30 fish and exposures were conducted in cylindrical cages of 0.3x1.0 m.

Fish in treatments 2 and 4 were exposed to genotype II for 24 h in the Williamson River, OR (N 42° 32.425, W 121° 52.787) a location where this genotype is predominant and genotype I is absent (Atkinson and Bartholomew 2010a; Hurst et al. 2012b). Water temperature during exposure was 16.5°C. The remaining treatments (1 and 3) were exposed to 18°C UV treated Willamette River water for 24 h at the SDL.

Exposure to genotype I was conducted in the laboratory where a pure parasite culture could be obtained. The exposure timeline was constrained by the availability of myxospores and polychaetes (Hurst and Bartholomew 2012a) and the activation time required for both the innate and adaptive immune responses (6-12 weeks; Sitjà-Bobadilla 2008). Thus, exposure to genotype I occurred 53 days after the initial exposure to genotype II, which was supposed to allow for activation of both the innate and adaptive immune response and coincided with genotype I production in the laboratory. At the SDL, each treatment replicate was placed in a separate cage within a flow-through 378 L tank containing either genotype I from cultured polychaete populations (treatments 3 and 4) or UV treated Willamette River water (treatments 1 and 2) for 24 h (Figure 1). Water temperatures were 21°C at the time of the second exposure.

Parasite exposure dose.−To calculate exposure dose per fish for field and laboratory challenges, 3x1 L of water were collected before and after each exposure. Water samples were filtered, parasite DNA was extracted and parasite density was measured by qPCR for each sample in duplicate (Hallett and Bartholomew 2006). Samples were considered positive if both duplicate wells fluoresced and were re-run if a difference of more than one cycle occurred between wells. One sample from the beginning and end of each exposure was tested to determine if parasite DNA detection was inhibited by other components in the water sample filtrate (Hallett and Bartholomew 2009). If inhibition occurred, samples were diluted 1:10 and re-run. Mean

parasite density for each exposure site and time was then multiplied by the velocity and exposure duration and divided by the number of fish (Ray et al. 2010). Velocity was 3 L/s in the field and 0.0083 L/s in the laboratory.

Assessment of Infection.− Immediately after each 24 h exposure, five fish from each replicate were euthanized with an overdose of tricaine methanesulfonate (MS-222 Argent Chemical Laboratories, Redmond, WA, USA) and the right half of the gill was excised, placed on ice, and stored at -20 °C to determine infection prevalence. The remaining fish were placed into 25 L tanks supplied with well water at 18°C. All fish were treated for external parasites and bacterial infections, fed daily and monitored for clinical disease signs according to Ray et al. (2010). Moribund fish were removed, euthanized as above and time to morbidity was recorded. Myxospores were counted by removing and weighing the intestine, flushing into a microcentrifuge tube using a pipette attached to one end of the intestine filled with 1 mL of tap water and using a hemocytometer at 200x magnification. A 25 mg aliquot of the harvested myxospores was stored at -20°C for genotype composition (see below). Fish remaining at the end of the experiment at 113 days were euthanized using an overdose of MS-222 and a 25 mg piece of intestine was collected and stored at -20°C to determine infection status in survivors (10 fish from each treatment were assayed).

DNA from gills and intestines was extracted and purified as in Hurst et al. (2014). After extraction, DNA from all samples was then directly tested for the presence of parasite DNA using qPCR as above. To create a standard curve for estimating the parasite DNA copy number in 0.1 g of host gill tissue, ten-fold serial dilutions of a synthetic parasite template were added to gill tissue (Hallett and Bartholomew 2006). Harvested myxospores from the intestine of 10

moribund fish in each of the treatments were sequenced to determine genotype composition (Hurst et al. 2014).

Infection prevalence and copy number were determined using gills from treatments 2 and 4 (genotype II; $n = 30$) and treatment 3 (genotype I; $n = 20$) at 1 day post exposure. S-PLUS version 8.2 (Tibco, Palo Alto, CA) was used to compare survival between treatments 3 and 4 and among treatment replicates using a Mantel-Cox test. One replicate from treatment 4 was lost when water flow to the tank was stopped and therefore was not included in the analyses. A one-way ANOVA with Tukey's test for highly significant differences was used to compare natural log transformed (for normality) myxospore counts among treatment replicates. If no differences were detected among replicates within a treatment, replicates were combined for analyses at the treatment level using a student's t-test. Differences were considered significant at $P < 0.05$.

<A>Results and Discussion

In this study, previous exposure to a less virulent genotype (II) of *C. shasta* did not prevent or limit mortality or myxospore production in Chinook salmon after a subsequent exposure to a more virulent genotype (I). There was no significant difference in survival between treatments 4 (57%) and 3 (48%; Mantel-Cox test$_1$ = 2.05, $p = 0.15$; Figure 2). There was also no difference in myxospore counts between these treatments (t-test, $t_5 = 0.462$, $p = 0.663$), with mean ± SE counts of 8173 ± 2108 and 7267 ± 612, respectively (Figure 3). Sequencing demonstrated that myxospores obtained from fish from both treatments were only of genotype I. Infection prevalence in surviving fish from treatments 3 and 4 was 50 and 40%, respectively. None of the fish in treatments 1 and 2 died and parasite DNA was not detected in these fish at the end of the study. These data indicate that prior exposure to genotype II does not reduce disease in

147 Chinook salmon. If genotype II was protective, we would have expected at least 74% survival
148 [the 50% infected with genotype II (45/90) in addition to 48% of the remaining naïve fish
149 exposed to genotype I (22/45)].

150 Differences in exposure conditions and in genotype virulence resulted in variations in
151 exposure dose and infection prevalence. The exposure dose of parasite genotype II in the field
152 was approximately 1.1×10^4 actinospores/fish and 50% of the fish became infected, as
153 determined by detection of parasite DNA in fish gills. Mean parasite copy number at 24 h was
154 4.3 (SE, 1.8) using our standard curve for gill tissue ($y = -3.35x + 38.40$; $r^2 = 0.997$). Parasite
155 genotype I exposure dose in the laboratory was lower, 9.2×10^1 actinospores/fish, but resulted in
156 a higher infection prevalence (100%) and a higher mean parasite copy number of 6.7×10^2 (SE,
157 48.5). Parasite DNA was not detected in the gills of control fish. The faster replication rate of
158 genotype I (author's unpublished data) combined with more optimal flow and temperature
159 conditions for the parasite in the slower flow of the laboratory challenge tanks (Ray et al. 2013)
160 likely contributed to the higher prevalence and intensity of genotype I infections.

161 Although these results suggest that previous exposure using a low virulence genotype is
162 not effective, modifications to exposure timing may yield different results. It is unknown
163 whether the adaptive response plays a protective role in the fish host's defense against *C. shasta*;
164 however, researchers found that rainbow trout infected with a less virulent parasite genotype of
165 *C. shasta* and surviving to three months had a 700 fold increase in IgT antibody levels compared
166 to unexposed fish (Zhang et al. 2010). Work with other myxozoans indicated that specific
167 antibodies were produced from 50 to 360 days post re-exposure by turbot (*Psetta maxima*) in
168 response to *E. scophthalmi* infection (Sitja-Bobadilla et al. 2007) and as early as 35 days post
169 exposure in rainbow trout infected with *M. cerebralis* (Hedrick et al. 1998). Thus, it seems likely

that the adaptive immune response to *C. shasta* infection would have been mounted during our experimental time frame, but a longer time between exposures may have allowed for increased production of a putative protective antibody.

The timing of the subsequent exposure to the more virulent genotype should also consider the timing of the hosts' innate immune response to the parasite, which may be elicited within hours to days of infection depending on temperature and stress (Sitjà-Bobadilla 2008; Gómez et al. 2014). For example, infections with *M. cerebralis* resulted in upregulation of immune relevant genes as early as five minutes post exposure (Severin and El-Matbouli 2007). Recently, Bjork et al. (2014) demonstrated that an inflammatory response to *C. shasta* is mounted within at least two weeks of exposure and is capable of resolving infection by 90 days. However, sampling was not conducted between the 25 and 90 day sample times, thus the infection may have been resolved sooner. This suggests that in this study infection with genotype II may have been resolved before exposure to genotype I occurred. A decrease in the interval between exposures may provide some short-term protection for the fish by taking advantage of the mounted inflammatory response.

The lack of protection after initial exposure to genotype II could be attributed to a parasite dose that did not elicit a host immune response and could be rectified by exposing fish to a higher dose of genotype II or lengthening the exposure time. Studies with *T. bryosalmonae* and *E. scopthalmi* demonstrated resistance to parasite reinfection only after lengthy continuous parasite exposures of 10 and 13 months, respectively (Foott and Hedrick 1987; Sitja-Bobadilla et al. 2007). Alternatively, immunization may be parasite genotype-specific, requiring initial exposure to the more virulent genotype to elicit an effective immune response. However, prior exposure to the more virulent genotype increases the probability of fish developing clinical

disease. To minimize this risk, fish could be exposed to a low dose of genotype I that enables fish to resolve the infection (Bjork et al. 2014). Despite the difficulties of working with a parasite that has a complex life cycle, immunization studies with *C. shasta* are worth pursuing because they may improve our understanding of how to implement management actions such as dam removal and/or fish reintroduction in a manner that could provide fish with some level of natural protection against the parasite.

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Figure Captions

Figure 1: Diagrammatic drawing of the experimental design of Chinook salmon exposure to two genotypes of *Ceratonova shasta*. Both exposures were for 24 h and included four treatments; control (treatment 1), genotype II only (treatment 2), genotype I only (treatment 3) and genotype II followed by genotype I (treatment 4). Exposure 1 took place in both the Williamson River, Oregon for treatments 2 and 4 and at the John L. Fryer Salmon Disease Laboratory at Oregon State University, Corvallis, Oregon (SDL) for treatments 1 and 3. Exposure 2 took place at the SDL for all four treatments. Five fish were euthanized immediately following both exposures for determination of infection.

Figure 2: Percent survival of Chinook salmon after exposure to *Ceratonova shasta* genotype I only (black line) or after exposure to genotype II followed by genotype I (gray line). Letters indicate statistical differences using a Mantel-Cox test.

Figure 3: Mean number of *Ceratonova shasta* myxospores produced in 0.1 g of intestinal tissue from moribund fish in treatments 3 (exposure to genotype I only) and 4 (exposure to genotype II then I). Error bars indicate standard error of the mean and letters indicate statistically significant differences using a student's t-test.