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ARTICLE

Effect of *Nanophyetus salmincola* and Bacterial Co-Infection on Mortality of Juvenile Chinook Salmon

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Abstract

The freshwater trematode *Nanophyetus salmincola* has been demonstrated to impair salmonid immune function and resistance to the marine pathogen *Vibrio anguillarum*, potentially resulting in ocean mortality. We examined whether infection by the parasite *N. salmincola* similarly increases mortality of juvenile Chinook Salmon *Oncorhynchus tshawytscha* when they are exposed to the freshwater pathogens *Flavobacterium columnare* or *Aeromonas salmonicida*, two bacteria that juvenile salmonids might encounter during their migration to the marine environment. We used a two-part experimental design where juvenile Chinook Salmon were first infected with *N. salmincola* through cohabitation with infected freshwater snails, *Juga* spp., and then challenged with either *F. columnare* or *A. salmonicida*. Cumulative percent mortality from *F. columnare* infection was higher in *N. salmincola*-parasitized fish than in nonparasitized fish. In contrast, cumulative percent mortality from *A. salmonicida* infection did not differ between *N. salmincola*-parasitized and nonparasitized groups. No mortalities were observed in the *N. salmincola*-parasitized-only and control groups from either challenge. Our study demonstrates that a relatively high mean intensity (>200 metacercariae per posterior kidney) of encysted *N. salmincola* metacercariae can alter the outcomes of bacterial infection in juvenile Chinook Salmon, which might have implications for disease in wild fish populations.

In a natural system, fish population dynamics can be regulated or limited by microparasites (viruses, bacteria, and protozoans) and such macroparasites as helminths and arthropods, etc. (Lester 1984; Sindermann 1987; Hershberger et al. 2006; Fujiwara et al. 2011). Effects of "co-infection" by micro- and macroparasites on host mortality, recovery, and parasite transmission rates can have far-reaching effects on disease dynamics in wild populations (Ezenwa and Jolles 2011), as parasites can interact within a single host (Pedersen and Fenton 2007). For example, chronic stress and suppressed host-immune function caused by an initial macroparasite infection can increase host susceptibility to a secondary microparasite infection (Graham 2008; Tort 2011). Experimental studies have shown that co-infection by various macro- and microparasites resulted in increased mortality of fingerling Channel Catfish *Ictalurus punctatus* (Labrie et al. 2004; Shoemaker et al. 2012; Xu et al. 2012) and higher bacterial loads in juvenile European Grayling *Thymallus thymallus* (Pylkkö et al. 2006). In addition to these synergistic effects, macroparasites can cause direct damage that facilitates secondary infection. For example, mechanical damage from attachment of the ectoparasite *Argulus coregoni* increased mortality from bacterial infection in Rainbow Trout *Oncorhynchus mykiss* (Bandilla et al. 2006).

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Understanding how macro- and microparasite co-infections might affect disease in wild salmonids is critical, given that disease might contribute to declining populations (Arkoosh et al. 2004; Ogut and Reno 2004; Fujiwara et al. 2011), and that these fish are commonly infected by multiple macro- or microparasites or both (Ferguson et al. 2011b; Van Gaest et al. 2011). In the U.S. Pacific Northwest (PNW), juvenile Chinook *O. tshawytscha* and Coho *O. kisutch* salmon are infected at high prevalence (60–80%) by the digenean trematode *Nanophyetus salmincola* (Arkoosh et al. 2004; Ferguson et al. 2011b). Many stocks of these species are listed as threatened or endangered under the Endangered Species Act (NMFS 2011), and how this parasite might interact with other pathogens to affect their survival is not fully understood. Salmonids become infected in freshwater when *N. salmincola* cercariae released from the first intermediate snail host penetrate and encyst as metacercariae (Bennington and Pratt 1960). Metacercariae can encyst in most fish tissues but have a propensity towards the posterior kidney and muscle (Jacobson et al. 2008). The encysted metacercariae survive until the fish is consumed by a definitive host, either a mammal or piscivorous bird (Schlegel et al. 1968). Studies on the pathogenicity of *N. salmincola* suggest that physical damage from cercarial penetration and migration can cause mortality in salmon fry exposed to a high number (e.g., 200 cercariae) at a time (Baldwin et al. 1967); if exposed over a period of time, however, the cercariae will encyst as metacercariae without lethal effects (Millemann and Knapp 1970). Nonetheless, field and laboratory studies indicate that encysted *N. salmincola* metacercariae can have negative, sublethal effects on swimming ability (Butler and Millemann 1971; Ferguson et al. 2012), growth (Sandell et al. 2015), osmoregulation enzymes (Ferguson et al. 2012), and immune function and disease resistance of juvenile salmonids when challenged with the marine bacteria *Vibrio anguillarum* (Jacobson et al. 2003).

Juvenile salmonids can accumulate *N. salmincola* metacercariae throughout their time spent in-river, wherever they overlap with the distribution of the freshwater *Juga* spp. snail host (Furnish 1990). Thus, if *N. salmincola* metacercariae infection increases susceptibility to freshwater bacterial infections, juvenile salmonids with a high intensity of infection with *N. salmincola* (number of metacercariae per infected fish) could be more likely to succumb to disease during their migration to the ocean. The bacteria *Flavobacterium columnare* and *Aeromonas salmonicida* ssp. *salmonicida* are ubiquitous in the freshwater environment (Austin and Austin 2012), are present in subclinical carrier fish or environmental reservoirs (Bullock and Stuckey 1975; Suomalainen et al. 2005a; Kunttu et al. 2012), and have been linked to mortality in hatchery and wild salmonid populations in the PNW (Becker and Fujihara 1978; Ogut and Reno 2004; Van Gaest et al. 2011) and therefore pose a potential threat to out-migrating juvenile salmonids.

To develop a better understanding of the effects that parasite-bacteria co-infection might have on the ecology of disease in wild salmonid populations, we simulated a co-infection situation under conditions experienced by salmonids at a temperature (18°C) typically recorded in PNW watersheds during late summer or early autumn (Billman et al. 2014), when conditions (high temperature, low flow) are favorable to epizootics of *F. columnare* or *A. salmonicida* (Wakabayashi 1991; Austin and Austin 2012). We hypothesize that juvenile Chinook Salmon having high intensities of encysted *N. salmincola* metacercariae will have greater mortality when challenged with bacterial pathogens than will fish that have no encysted *N. salmincola*. Here, we present results from two bacterial challenges designed to investigate the effects of *N. salmincola* infection on mortality from two freshwater bacteria, *F. columnare* and *A. salmonicida*.

METHODS

Fish hosts.—Approximately 1,000 juvenile spring Chinook Salmon (~1.5 g each) were obtained from Marion Forks fish hatchery (Idanha, Oregon) in June 2013. Fish were transported in aerated 95-L transport tanks with 11°C water to the John L. Fryer Salmon Disease Laboratory (SDL; Corvallis, Oregon), where they were held in a 379-L circular, aerated, flow-through tank supplied with pathogen-free well water (13°C) for 6 weeks prior to initiation of the study. Unless otherwise noted, all experiments were conducted in aerated, flow-through tanks supplied with 13°C water flowing at 2 L/min. Fish were fed 1–2% body weight/d with appropriately sized fish feed (Bio-Oregon, Inc., Warrenton, Oregon but not during 24 h before and after bacterial challenges. Prior to bacterial challenge, gill and kidney tissues from 40 randomly sampled fish were tested for bacterial pathogens and metacercariae as described below. Results for all fish were negative. All procedures utilizing fish were approved by the Oregon State University (OSU) Institutional Animal Care and Use Committee.

Source of N. salmincola.—Freshwater *Juga* spp. snails were periodically collected from coastal rivers (Siletz, Alsea rivers), Willamette River tributaries (Luckiamute, McKenzie, South Santiam rivers), and the Willamette River main stem near the SDL from June to September 2013. Collected snails were transported to the SDL and placed in single wells of a 12-well tissue culture plate (1 snail per well). Wells were filled with 13°C well water and a piece of organic lettuce and were incubated in the dark at room temperature (21°C) overnight. The next day, wells were examined for *N. salmincola* cercariae, the stage infective to fish, using a binocular dissection microscope at 50 × magnification. Any snail shedding the distinctive *N. salmincola* cercariae was moved to a 100-L flow-through tank at 13°C where they were maintained for up to 8 weeks prior to fish exposure.

Exposures to cercariae were conducted in 100-L tanks, in which fish were randomly assigned to either the control or the *N. salmincola* treatment group. This randomization was performed by transferring groups of five fish at a time to each tank according to a randomly generated sequence of tank numbers. Treatments were replicated four times for a total of eight tanks. Fish ($n = 125$ per tank) cohabitated with *N. salmincola*-infected

Juga spp. snails over a 10-week period. Control fish ($n = 125$ per tank) were held in identical tanks without snails. At the start of the cohabitation period, each *N. salmincola* treatment tank contained 20 *N. salmincola*-infected snails; to increase *N. salmincola* transmission, 10 additional infected snails were added weekly to each tank until there were 60 snails/tank (as per Jacobson et al. 2003). Snails were allowed to roam the tank freely and were fed weekly with organic lettuce. Five fish from each tank were subsampled at 2, 6, 8, and 10 weeks to quantify progression of *N. salmincola* infections. All infected snails were screened after the cohabitation period and were observed to still be releasing *N. salmincola* cercariae.

Bacteria and culture conditions.—Isolates of *F. columnare* and *A. salmonicida* were previously lyophilized and kept at −20°C until use. The *F. columnare* isolate was originally isolated from a gill lesion of a juvenile spring Chinook Salmon with columnaris disease at Dexter Ponds Rearing Facility (Lowell, Oregon). The *A. salmonicida* isolate was originally isolated from the kidney of a juvenile winter Steelhead Trout *O. mykiss* from Rock Creek Hatchery (Idleyld Park, Oregon) that had furunculosis.

A stock of *F. columnare* was created by rehydrating the lyophilized bacteria with 2 mL of tryptone yeast extract salts (TYES; 4 g/L tryptone, 0.4 g/L yeast extract, 0.5 g/L calcium chloride, 0.5 g/L magnesium sulfate, pH 7.2; Holt et al. 1993) broth and incubating on TYES agar plates for 72 h at 21°C. Individual colonies were then removed and inoculated in 50 mL of tryptone yeast infusion broth (TYI; 4 g/L tryptone, 30 mL/L yeast infusion, pH 7.2; Pacha and Ordal 1970) for 24 h at 21°C on a shaker set to 140 rpm. A 1-mL aliquot was mixed with 200 mL/L glycerol and maintained at −80°C until used for bacterial challenges. Prior to challenges, *F. columnare* was cultured as previously described. A 50-mL culture was used to inoculate 500-mL culture flasks with TYI broth. After 24 h, absorbance at 525 nm (A_{525}) was measured using a Spectronic 20 (Thermo Scientific) and adjusted to 1.0 *A* with sterile TYI broth.

We cultured *A. salmonicida* in the same manner, but inoculating in tryptic soy broth (TSB; BD Biosciences) at 18°C on a shaker set to 200 rpm. We were unable to maintain a viable stock of *A. salmonicida* at −80°C. Therefore, we inoculated rehydrated *A. salmonicida* colonies, cultured them as described above, and used them for a challenge to determine the dose required to induce 50% mortality (LD50). Colonies of *A. salmonicida* were reisolated from kidney tissue of three fish that died during the LD50 challenge; these isolates were combined and cultured as previously described and used for the *A. salmonicida* co-infection challenge. Culture purity was confirmed by gram staining and incubation on agar plates. Cultures of *F. columnare* were inoculated on TYES agar plates and incubated for 72 h at 21°C. The bacteria grew as yellow, adhesive, rhizoid colonies that were motile, long, thin, gram-negative rods. Cultures of *A. salmonicida* were inoculated on tryptic soy agar (TSA; BD Biosciences) and incubated for 5 d at 18°C. The bacteria produced a brown water-soluble pigment on TSA and were catalase- and oxidase-positive, nonmotile, gram-negative rods. A sample of each culture with 1.0 A_{525} was taken and plated (in triplicate) to determine the colony forming units (cfu)/mL and to calculate the bacterial concentration for each challenge.

LD50 determination.—Preliminary challenges were conducted to determine what dose induced 50% mortality, modifying the challenge methods for each bacterial isolate. Fish (30–35 g) were transferred to 25-L tanks and held for 3 d prior to both challenges. For fish challenged with *F. columnare*, the water temperature was gradually increased from 13°C to 18°C over a 4-h period after transfer. Doses of *F. columnare* in the current study were based on Holt et al. (1975), who reported 52% mortality in juvenile spring Chinook Salmon challenged with 3–6 × 10^6 cfu/mL of a similar isolate at 17.8°C. Duplicate groups of 10 fish were challenged with concentrations of *F. columnare* at 1.5 × 10^6, 2.0 × 10^6, and 4.5 × 10^6 cfu/mL, using a 60-min static immersion challenge at 18°C with aeration. The approximate LD50 for this *F. columnare* isolate corresponded to a 2.0 × 10^6 cfu/mL concentration (55% mortality; 5 or 6 of 10 fish, died in each tank challenged at this concentration), achieved by decreasing the tank volume to 19 L and diluting 40 mL of 1.0 A_{525} culture in 1 L of water before adding this solution directly to the tank. After 60 min, the water supply was resumed.

Prior to the *A. salmonicida* LD50 challenge, fish were transferred to 25-L tanks but the water temperature remained at 13°C for the 3-d acclimatization. Duplicate groups of eight fish were challenged with low (3.6 × 10^6 cfu/mL) and high (2.9 × 10^7 cfu/mL) concentrations using a 30-min static immersion challenge with aeration at 13°C. To increase the likelihood of *A. salmonicida* transmission, we additionally thermally stressed the fish by resuming the water flow following the challenge at 18°C. The approximate LD50 for this *A. salmonicida* isolate corresponded to the 3.6 × 10^6 cfu/mL concentration (56.25% mortality; 4 and 5 out of 8 fish died in each tank challenged at this concentration) and was achieved by lowering the tank volume to 19 L and diluting 30 mL of 1.0 A_{525} culture in 1 L of water before adding this solution directly to the tank. After 30 min, the water supply was resumed.

Flavobacterium columnare co-infection challenge.—After 20 weeks post-*N. salmincola* exposure, fish (46.9 ± 1.2 g; mean ± SE) were randomly assigned to 25-L tanks ($n = 10$ fish per tank) with a 2 × 2 factorial design, in which tanks containing *N. salmincola*-parasitized fish or control fish were either challenged with *F. columnare* or treated with equal volumes of sterile TYI broth. Treatments were replicated three times for a total of 12 tanks. Treatments included noninfected control (C), *N. salmincola* only (N), *F. columnare* only (Fc), and *N. salmincola* + *F. columnare* co-infection (N + Fc). After transfer to 25-L tanks, the water temperature was gradually increased from 13°C to 18°C over a 4-h period and fish were held at this temperature for 3 d before the *F. columnare* challenge. Fish in the Fc and N + Fc treatments were challenged with 2.0 × 10^6 cfu/mL of *F. columnare* as detailed above. Fish in

the control and *N. salmincola*-only treatments were treated in the same manner with diluted, sterile TYI broth.

Aeromonas salmonicida co-infection challenge.—Control and *N. salmincola*-parasitized fish (41.9 ± 2.0 g) were randomly assigned to new 25-L tanks ($n = 10$ fish per tank) in the same 2 × 2 factorial design as described above. Treatments included noninfected control (C), *N. salmincola* only (N), *A. salmonicida* only (As), and *N. salmincola* + *A. salmonicida* co-infection (N + As). Fish were held at 13°C for 3 d before the *A. salmonicida* challenge; upon resumption of the water flow after the challenge, the inflow was supplied at 18°C. The As and N + As treatments were challenged with 1.2×10^6 cfu/mL of *A. salmonicida* as described above. The control and *N. salmincola*-only treatments were treated in the same manner with diluted, sterile TSB.

Fish monitoring.—Following all bacteria challenges, fish were monitored and mortalities were removed every 12 h. Experiments were terminated after mortality had ceased for at least 7 consecutive days. Any moribund fish during the observation period and all fish surviving at the termination of the experiment were euthanized with 250 mg/L buffered tricaine methanesulfonate (MS-222; Argent Chemical Laboratories). Clinical signs, such as darkening, lethargy, loss of equilibrium, lack of feeding, and characteristic lesions (e.g., yellow areas of necrosis or furuncles) were recorded along with weight (g) and fork length (mm) for evaluation of condition factor (weight/length^3). The posterior half of kidneys from each fish was collected in Stomacher bags (Seward, Ltd.) and frozen at −20°C for later quantification of *N. salmincola* metacercariae.

Necropsy and diagnostic confirmation.—For *F. columnare*–challenged fish, gill and mid-kidney tissues were streaked onto separate TYES agar plates. For *A. salmonicida*–challenged fish, mid-kidney tissue was streaked onto TSA plates. All plates were incubated at 21°C and examined at 3 and 7 d for bacterial growth and phenotypic characteristics. Presumptive identification of bacteria was based on observation of characteristic lesions and colony morphology (AFS–FHS 2014). To quantify *N. salmincola* metacercariae, the posterior kidney tissues were thawed and then squashed within the Stomacher bag using a glass slide and examined using a binocular dissection microscope at 50–100 × magnification (Ferguson et al. 2010). Parasite intensity was quantified within the posterior half of the kidney, given that consistently high concentrations of *N. salmincola* metacercariae are known to encyst in this tissue (Jacobson et al. 2008). Mean intensity was defined as the average number of *N. salmincola* metacercariae per posterior kidney among all the infected fish.

Statistical analysis.—Logistic regression models with a random effect for tank were used to fit the cumulative percent mortality data from each challenge. This model assumes that mortality and survivor data follow a binomial distribution (Jacobson et al. 2003). A likelihood ratio test was used to evaluate whether co-infection mortality differed from the baseline mortality from bacteria alone. Mean *N. salmincola* intensity of each co-infection treatment (N + Fc, N + As) was compared between bacterial challenges using a Student's two-sample *t*-test. For each bacterial challenge, mean *N. salmincola* intensity was compared among tanks containing *N. salmincola*-parasitized fish by using an analysis of variance (ANOVA) and was compared between survivors and mortalities within each co-infection treatment by using a Student's two-sample *t*-test. Mean values were considered significantly different at $P < 0.05$. These analyses were performed using R version 3.1.1 (R Core Team 2014).

RESULTS

Nanophyetus salmincola *Exposures*

Intensity of *N. salmincola* was not associated ($F_{3, 116} = 6.60$, $R^2 = 0.124$, $P < 0.001$) with fish length, weight, or condition factor in either challenge. Mean *N. salmincola* intensity (metacercariae per posterior kidney) in the N + Fc treatment (249.7 ± 19.2) was not significantly different ($t_{58} = 1.883$, $P = 0.175$) from that in the N + As treatment (215.6 ± 15.7).

Flavobacterium columnare *Challenge*

Mean cumulative mortality was significantly higher in the N + Fc treatment (80.0% ± 0.0%; mean ± SE) than in the Fc treatment (50.0% ± 10.0%) at the conclusion of the experiment ($\chi^2 = 5.63$, $P = 0.018$; Table 1). The majority of deaths occurred 1–2 d postchallenge (dpc; Figure 1). Colonies of *F. columnare* were isolated from the gills of all mortalities ($n = 39$) during the experimental period, but *F. columnare* was not isolated from kidney tissue of these fish. Pale yellow areas of necrosis characteristic of columnaris infection were observed on gill tissues in only three mortalities from the Fc ($n = 1$) and N + Fc ($n = 2$) treatments. Colonies of *F. columnare* were not detected in the gills or kidney tissue from any surviving fish at 10 dpc.

TABLE 1. Cumulative percent mortality of juvenile Chinook Salmon as a result of *F. columnare* or *A. salmonicida* challenge of *N. salmincola*-parasitized fish (co-infection) and nonparasitized fish (bacteria only). Treatments without bacteria of *N. salmincola*-parasitized fish and nonparasitized (negative control) fish did not have any mortality. Shown are the number of mortalities per the total number of fish in each study; percentages of mortalities are in parentheses. The mean cumulative percent mortality is shown ± 1 SE.

	Flavobacterium columnare		*Aeromonas salmonicida*	
	Bacteria only (Fc)	Co-infection (N + Fc)	Bacteria only (As)	Co-infection (N + As)
Replicate				
1	4/10 (40%)	8/10 (80%)	6/10 (60%)	5/10 (50%)
2	4/10 (40%)	8/10 (80%)	6/10 (60%)	3/10 (30%)
3	7/10 (70%)	8/10 (80%)	2/10 (20%)	8/10 (80%)
Mean	50.0% ± 10%	80.0% ± 0.0%[a]	46.7% ± 13.3%	53.3% ± 14.5%

[a]Indicates significant difference from bacteria only group ($P < 0.05$).

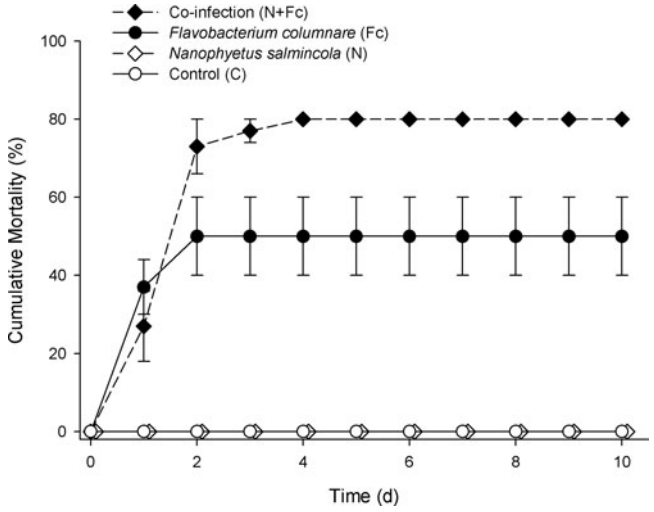

FIGURE 1. Cumulative percent mortality (mean ± SE) of juvenile Chinook Salmon in one of four treatments in the *F. columnare* challenge: encysted *N. salmincola* metacercariae challenged with *F. columnare* (N + Fc), *F. columnare* (Fc) only, encysted *N. salmincola* (N) only, and a noninfected control (C). The difference in cumulative percent mortality between the co-infection and Fc-only groups on day 10 were significant ($P = 0.018$).

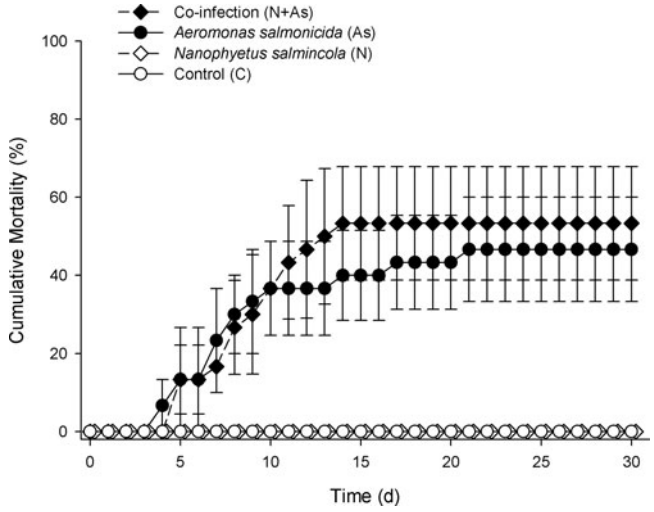

FIGURE 2. Cumulative percent mortality (mean ± SE) of juvenile Chinook Salmon in one of four treatments in the *A. salmonicida* challenge: encysted *N. salmincola* metacercariae challenged with *A. salmonicida* (N + As), *A. salmonicida* (As) only, encysted *N. salmincola* (N) only, and a noninfected control (C). The difference in cumulative percent mortality between the co-infection and As-only groups on day 30 was not significant.

No mortality was observed and bacteria were not isolated in fish from the C or N treatments. All *N. salmincola*-parasitized fish examined prior to bacterial challenge had metacercariae encysted in the posterior kidney (intensity range, 111–526; [mean ± SE] 245.5 ± 10.0). Overall, there were no significant differences in mean *N. salmincola* intensity among tanks containing *N. salmincola*-parasitized fish ($F_{5, 54} = 0.066$, $P = 0.997$). Mean *N. salmincola* intensity did not significantly differ ($t_{28} = 1.152$, $P = 0.259$) between survivors (293.7 ± 0.4) and mortalities (239.7 ± 0.2) within the co-infection treatment. There were no significant differences in mean weight, length, or condition factor between treatment groups in this challenge.

Aeromonas salmonicida *Challenge*

Mean cumulative percent mortality in the N + As treatment (53.3% ± 14.5%) was marginally higher than observed in the As treatment (46.7% ± 13.3%), but these were not significantly different ($\chi^2 = 0.18$, $P = 0.674$) at the conclusion of the experiment. Mortality occurred from 4 to 21 dpc (Figure 2). Colonies of *A. salmonicida* were isolated from the kidneys of all mortalities in *A. salmonicida* challenge tanks during the experimental period. External furuncle lesions were observed in all mortalities ($n = 30$). Colonies of *A. salmonicida* were not detected in the kidneys of any surviving fish at 30 dpc. No mortality was observed and bacteria were not isolated in fish in the C or N treatments. Prior to bacterial challenge, all *N. salmincola*-parasitized fish had metacercariae encysted in the posterior kidney (range, 82–439; mean intensity, 239.2 ± 13.2). Overall, there were no significant differences between mean *N. salmincola* intensity among tanks containing *N. salmincola*-parasitized fish ($F_{5, 54} = 1.366$, $P = 0.252$). Mean *N. salmincola* intensity did not significantly differ ($t = 1.800$, $P = 0.083$) between survivors (244.7 ± 0.2) and mortalities (190.2 ± 0.2) within the co-infection treatment. There were no significant differences in mean weight, length, or condition factor between treatment groups.

DISCUSSION

Using a laboratory challenge method, we investigated the effects of *N. salmincola* parasitism on outcomes of infection by *F. columnare* and *A. salmonicida* in juvenile Chinook Salmon. Cumulative percent mortality from *F. columnare* infection was higher in *N. salmincola*-parasitized than in nonparasitized juvenile Chinook Salmon. In contrast, cumulative percent mortality from *A. salmonicida* infection did not differ between *N. salmincola*-parasitized and nonparasitized groups. Our study demonstrates that the outcomes of some bacterial infections in juvenile Chinook Salmon can be altered by previous infection with *N. salmincola*, which might have important implications for disease in wild salmonid populations throughout the PNW.

Our results support a previous study that showed encysted *N. salmincola* metacercariae can increase mortality from a marine bacterial infection in juvenile Chinook Salmon (Jacobson et al. 2003). Few studies have investigated co-infection effects of digenean metacercariae and bacterial infections on fish despite the common cooccurrence of these pathogens in juvenile salmonids (e.g., Sandell et al. 2015) and other fishes (e.g., Xu et al. 2007). Most studies involving larval digenean bacteria co-infection in fish have shown that increased mortality or bacterial invasion occurs when fish are concurrently challenged with cercariae and bacteria (e.g., *Bolbophorus* spp. and the bac-

teria *Edwardsiella ictaluri,* Labrie et al. 2004; *Diplostomum spathaceum* and atypical *A. salmonicida,* Pylkkö et al. 2006) but once encysted, digenean metacercariae do not affect the outcome of bacterial infections (e.g., *D. spathaceum* and *F. columnare,* Suomalainen et al. 2005b), suggesting the mechanism might be associated with damage or stress-related responses to parasite penetration and initial stages of parasite migration. However, our finding that encysted metacercariae affected the outcome of *F. columnare* infections in Chinook salmon suggests that certain digenean metacercariae can have chronic, sublethal effects on host susceptibility. The former studies involved metacercariae that primarily encyst in the skin (*Bolbophorus* spp.) and eye (*D. spathaceum*), whereas *N. salmincola* encysts in high concentrations in the posterior kidney (Millemann and Knapp 1970), indicating that differences in larval digenean co-infection outcomes on host mortality might depend on the tissue(s) infected and host response to the parasite (Whyte 2007).

We found that a mean intensity of >200 metacercariae per posterior kidney of encysted *N. salmincola* was associated with higher mortality when Chinook Salmon were challenged with *F. columnare*. Similarly, Jacobson et al. (2003) reported a relatively high mean *N. salmincola* intensity of 394 metacercariae (range, 257–504) in co-infected juvenile Chinook Salmon from their disease challenge. This suggests that a relatively high *N. salmincola* intensity (e.g., ∼200 metacercariae) might have detectable effects of co-infection on mortality in a juvenile Chinook Salmon population. Macroparasites typically have an aggregated distribution in the wild, with few to no parasites in the majority of hosts and high parasite intensities in a small percentage of hosts (Lester 1984; Ferguson et al. 2011a). For example, mean *N. salmincola* intensities in wild juvenile Chinook Salmon caught off the coast of Oregon and Washington ranged from 18 to 73 metacercariae, but these fish included a small proportion (∼5%) of individuals with intensities exceeding 200 metacercariae (Jacobson et al. 2008; Sandell et al. 2015). Thus, it is possible the effects of co-infection on mortality occur only in the heavily *N. salmincola*-infected proportion of the out-migrating population.

The contrast in effects of *N. salmincola* on mortality between the two bacterial challenges might be a result of dissimilarities in bacteria pathogenesis and the relative virulence of isolates. Virulent *F. columnare* isolates are associated with attachment and erosion of the gills and skin (Decostere et al. 1999), whereas *A. salmonicida* can affect many internal organs, presenting disease as an internal hemorrhagic septicemia (Austin and Austin 2012). In this study, challenge with *F. columnare* caused mortality within 2 d and bacteria were isolated primarily from the gills, indicating the infection did not become systemic. Gross pathology is usually limited in fish infected with a highly virulent *F. columnare* isolate, which can cause mortality without macroscopic evidence of gill damage (Pacha and Ordal 1970; Austin and Austin 2012). Other studies have shown differences in virulence among *F. columnare* strains (Suomalainen et al. 2006; Shoemaker et al. 2008; LaFrentz et al. 2012) and have demonstrated that differences among isolates can change the effect of co-infection with the ciliate *Ichthyophthirius multifiliis* on bacterial load and host mortality (Xu et al. 2014). In contrast, mortality following the *A. salmonicida* challenge was lower and progressed over a longer period (4–21 dpc), despite the use of a temperature stressor to facilitate transmission. Bacteria isolated from kidney tissue indicate a systemic infection, reflecting differences in pathogenesis between our two challenge species. However, the lack of a synergistic effect following *A. salmonicida* challenge in the present study contrasts with the increased mortality observed in a co-infection challenge with *N. salmincola* and *V. anguillarum* (Jacobson et al. 2003), a bacteria that also causes internal hemorrhagic septicemia in salmonids. In this case, dissimilarity in experimental conditions might explain the differences in mortality outcomes. Jacobson et al. (2003) challenged juvenile Chinook Salmon following acclimatization to saltwater, a physiological change that can contribute to a greater immunocompromised state (Maule et al. 1989; Tort 2011) and might have altered the effect *N. salmincola* had on the host.

Immunosuppression from encysted *N. salmincola* has been suggested to increase susceptibility to infection and disease, as metacercariae can decrease B cell function in parasitized juvenile Chinook Salmon (Jacobson et al. 2003). Immunosuppression might also result from chronic stress (Salonius and Iwama 1993; Tort 2011), as histopathology of *N. salmincola* infection indicates mild, chronic inflammation around encysted metacercariae (Ferguson et al. 2010), and host condition deteriorates as an active immune response depletes energetic resources (Barber and Wright 2005). Given the evidence for immunosuppressive effects of *N. salmincola*, it was unexpected that a general effect of co-infection on mortality was not observed in both challenges. However, sublethal effects are another possible outcome of co-infections, as reduced growth in some juvenile Chinook Salmon off the coast of Oregon and Washington was associated with co-infections of *N. salmincola* and the bacteria *Renibacterium salmoninarum* (Sandell et al. 2015). Therefore, the mechanism(s) underlying pathogenicity of co-infections probably are complex and vary with different macro- and microparasites.

An increased susceptibility of juvenile salmonids to freshwater and marine bacterial pathogens suggests the importance of *N. salmincola* to the ecology of disease in out-migrating wild juvenile Chinook Salmon throughout the PNW. For example, wild subyearling and yearling Chinook Salmon first encounter the trematode while rearing in the upper Willamette River and natal tributaries (Friesen et al. 2007; Van Gaest et al. 2011); there, they might accumulate relatively high *N. salmincola* intensities before entering the ocean, given that *Juga* spp. snails are distributed throughout the Willamette River main stem and tributaries (Furnish 1990) and infected snails have been shown to release *N. salmincola* cercariae as temperatures reach 10°C in the spring (Gebhardt et al. 1966). As the juvenile salmon migrate into the main stem, they can encounter increasing densities of bacterial pathogens, particularly when fish densities and river temperatures are high (Becker and Fujihara 1978; Van

Gaest et al. 2011; Kent et al. 2013), therefore increasing the risk of infection or mortality for co-infected individuals during migration.

In conclusion, we have demonstrated that encysted *N. salmincola* metacercariae can significantly alter the outcome of *F. columnare* infection in juvenile Chinook Salmon. This provides further evidence that infections with *N. salmincola* metacercariae and this bacterial agent might lead to higher mortalities in co-infected Chinook Salmon populations. However, the effects of encysted *N. salmincola* metacercariae apparently are more complex than we initially hypothesized, given that a co-infection effect on mortality was not observed in both bacterial challenges. Further investigation of immune responses to *N. salmincola* metacercariae and co-infection is needed to explain within-host interaction and might help predict other macro- and microparasite co-infection outcomes in juvenile Chinook Salmon. This study suggests the importance of co-infection to the ecology of disease of out-migrating wild juvenile Chinook Salmon, such that incorporating co-infection into epidemiological models might elucidate how *N. salmincola* co-infection influences disease dynamics in wild salmonids.

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