DISSERTATION

BACTERIAL COLDWATER DISEASE INVESTIGATIONS

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ABSTRACT

BACTERIAL COLDWATER DISEASE INVESTIGATIONS

Global fish production has increased steadily since the 1950's, with the number of wild-captured fish reaching a plateau at an all-time high and the number of fish produced by aquaculture increasing in the early 2000s. In recent years, aquaculture accounts for over 60% of global fish production and is valued at about $250 billion US dollars. Within the United States, 169 million tons of trout are produced for food, restoration, and conservation practices and are worth more than $230 million US dollars. Trout production losses within the United States have averaged roughly 30 million trout per year and disease explains upwards of 90% of these losses.

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD), is found in cultured and wild fishes worldwide and causes significant infection in captive salmonid populations. Mortality associated with infections can be as high as 90% depending on water temperature and developmental stage of the host. Due to mortalities associated with BCWD, outbreaks can result in massive economic losses to producers of salmon and Rainbow Trout *Oncorhynchus mykiss*. As a result, BCWD is considered one of the most important hatchery diseases in the world. Infections typically affect age-0 salmonids but can also affect larger and older fish. Infected fish show a broad range of clinical signs of disease such as discoloration of the adipose fin, lesions, spiral swimming behavior, "blacktail", spinal deformities, and pale or necrotic gills.

Management options to treat infection due to *F. psychrophilum* consist of preventing infections by reducing crowding, handling, and physical damage, and by maintaining high water quality, as well as use of antibiotics, vaccines, or resistant fish hosts. The most common management option is the use of antibiotics. Oxytetracycline has been used worldwide, and amoxicillin and oxolinic acid have been used throughout Europe. However, several studies suggest that antimicrobial resistance is occurring in treated populations. Vaccines are a possible management option and though development of a vaccine has been

attempted, none are currently commercially available. Due to concerns about antibiotic resistance and the lack of a vaccine, other strategies to prevent *F. psychrophilum* infections warranted investigation such as using genetically resistant hosts. In 2005, the US Department of Agriculture-Agricultural Research Service's (USDA-ARS) National Center for Cool and Cold Water Aquaculture (NCCWA) developed a program to create a Rainbow Trout strain that was genetically resistant to *F. psychrophilum*. The resulting Rainbow Trout exhibit genetic resistance to *F. psychrophilum* and are currently being using within various aquaculture settings.

A similar approach of using genetically resistant hosts has been used to manage Rainbow Trout in the presence of the whirling disease parasite, *Myxobolus cerebralis*. Researchers at Colorado Parks and Wildlife (CPW) developed *M. cerebralis*-resistant strains of Rainbow Trout by crossing a domesticated, *M. cerebralis*-resistant strain with wild strains of Rainbow Trout. These crosses result in Rainbow Trout that are resistant to *M. cerebralis* and retain important wild characteristics, including the ability to survive and breed in the wild.

Currently in Colorado hatcheries, it is common practice to rear fish at high densities to maximize the number of fish available to stock for recreational fishing. *Flavobacterium psychrophilum* is managed by using *F. psychrophilum*-resistant Rainbow Trout and antibiotics and *M. cerebralis* is managed by using *M. cerebralis*-resistant Rainbow Trout. This results in Rainbow Trout that are resistant to *F. psychrophilum* in the hatchery but not *M. cerebralis* in the wild or Rainbow Trout that are resistant to *M. cerebralis* but not *F. psychrophilum*. Currently there is no Rainbow Trout that is resistant to both *F. psychrophilum* and *M. cerebralis*. Therefore, it is important to understand the ecological effects or benefits of rearing fish at lower densities to manage *F. psychrophilum* infections and determine if it is possible to develop dual resistance to both *F. psychrophilum* and *M. cerebralis*.

Three chapters comprise my dissertation with the goal of investigating Rainbow Trout management options in the face of *F. psychrophilum*. Chapter 1 describes the factors and the differences among those factors that affect mortality when Rainbow Trout are exposed to *F. psychrophilum* within a laboratory setting. Chapter 2 investigates genetic resistance of Rainbow Trout first-generation and multi-

generation crosses to both *F. psychrophilum* and *M. cerebralis* to determine if resistance to both pathogens is possible. In Chapter 3, I investigated the ecological implications of rearing Rainbow Trout at high rearing densities and on different feed types to determine their effects on Rainbow Trout survival post-stocking.

In Chapter 1, I reviewed and conducted a Bayesian meta-analysis of the *F. psychrophilum* literature to determine what factors affect mortality when Rainbow Trout are exposed to *F. psychrophilum* in the laboratory. I examined how bacterial dose, culture time, exposure method, bacterial isolate, and fish weight impacted mortality. I identified studies published in peer reviewed journals utilizing Web of Science, Academic Search Premier, and Google Scholar. Mortality was analyzed use a hierarchical Bayesian binomial beta regression model using Just Another Gibbs Sampler (JAGS) within program R. Review of the literature resulted in 22 manuscripts that contributed a total of 132 data points with 24 covariates used in the analysis. The meta-analysis shows that mortality from *F. psychrophilum* is variable among studies published since 1999, despite advances in culture methods and individual laboratory standardization of experimental methods. Injection produces more mortality than bath immersion, bacterial isolates differ in their effect on mortality, and bacterial dose is an important aspect affecting mortality due to *F. psychrophilum* exposures.

In Chapter 2, I conducted two experiments to assess dual pathogen resistance of first-generation and multi-generation crosses (F1) of Rainbow Trout created by crossing *M. cerebralis*- and *F. psychrophilum*-resistant strains. In the first experiment, I exposed two Rainbow Trout strains and one Rainbow Trout cross (German Rainbow x Harrison Lake Rainbow, GR x HL; *psychrophilum*-resistant Rainbow Trout, PRR; and GR x HL x PRR, GHP) to six different treatments: control (no exposure), mock injection, *F. psychrophilum* only, *M. cerebralis* only, *F. psychrophilum* followed by *M. cerebralis*, and *M. cerebralis* followed by *F. psychrophilum*. Rainbow Trout were exposed to *F. psychrophilum* with a dose of $8.8\text{x}10^6$ colony forming units per milliliter (CFU/mL) using subcutaneous injections and exposed to *M. cerebralis* using a static bath of 2,000 triactinomyxons per fish. Results indicated that GHP fish were not resistant to either pathogen. Dual resistance may be achieved if different parent strains

are used to create different F1 crosses. In the second experiment I exposed five Rainbow Trout strains and four Rainbow Trout crosses (GR; HL; PRR; USDA-ARS *F. psychrophilum*-resistant Rainbow Trout, ARS-Fp-R; USDA-ARS susceptible line, S-Line; HL x PRR; HL x ARS-Fp-R; GR x PRR; and GR x ARS-Fp-R) to *F. psychrophilum*. The second experiment indicated that there was at least one Rainbow Trout F1 cross, HL x PRR, that is *F. psychrophilum*-resistant. Although it appears that dual resistance may be possible with some strains, the lack of response in others indicates that dual resistance may be difficult to develop. However, some strains may be good candidates and a combination of crossing and selective breeding may be capable of achieving dual resistance.

In Chapter 3, my goal was to experimentally determine whether and to what extent rearing density and feed affect post-stocking survival of Rainbow Trout fry when stocked into a put-grow-and take fishery. German Rainbow x Harrison Lake Rainbow Trout, GR x HL, were raised for three months in the hatchery at two densities (high = 1,400 fish/ft^3 – 350 fish/ft^3, rearing index 2.0; and low = 350 fish/ft^3 – 87.5 fish/ft^3, rearing index 0.5) and fed two commercially available feeds (Bio Oregon and Rangen). Each treatment (4) had two replicates. Fish were tagged with passive integrated transponder (PIT) tags and stocked into Parvin Lake, Red Feather Lakes, Colorado. Recaptures of tagged fish occurred every two weeks for the first two-months, and again at seven- and 12-months post-stocking. At the time of stocking there were no differences in Fulton's Condition Factor, total length, or weight for each treatment. However, at the time of stocking, hepatosomatic index (HSI) was higher for the fish raised at low density and fed Bio Oregon feed. Recapture data indicated that there was no difference in Fulton's condition factor and HSI post-stocking and apparent survival of stocked fish was higher for fish raised at low density in the hatchery but was not affected by feed type. My study suggest that rearing density affects fingerling Rainbow Trout post-stocking survival. Higher number of fish stocked due to fish being reared at higher densities within the hatchery did not result in more total fish after a year in the lake compared to the low-density treatment. Raising fish at high density uses increased resources and may not provide any additional catchable fish for anglers.

In summary, exposure experiments are an important aspect to BCWD research. There are important factors such as bacterial dose, which bacterial isolate should be used, and how fish are exposed to the bacteria that affects mortality. Moving forward, *F. psychrophilum* resistance is a promising management option, however, depending on the desired traits, the correct parental strains need to be used to achieve *F. psychrophilum* resistance. Finally, preventative measures such as rearing Rainbow Trout at lower densities could have profound ecological impacts on survival post-stocking and reduce factors associated with BCWD outbreaks.

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CHAPTER 1: FACTORS AFFECTING FLAVOBACTERIUM PSYCHROPHILUM EXPERMENTAL INFECTIONS: A META-ANALYSIS OF THE LITERATURE

INTRODUCTION

Emerging infectious diseases are a major concern in the production, management, and conservation of fish and wildlife populations. These diseases can contribute to the decline and extinction of wild and domesticated animal populations (e.g., Vincent 1996; Berger et al. 1998; Daszak et al. 1999; Michel et al. 1999; Muths et al. 2003; Skerratt et al. 2007; Robinson et al. 2010). Infectious bacterial pathogens are particularly concerning in an aquaculture context and can be economically devasting. Bacterial diseases affect many economically important aquaculture fishes such as salmon, trout, tilapia, and catfish (Klesius and Pridgeon 2011; Pridgeon and Klesius 2012), and it is estimated that billions of US dollars per year are lost due to disease (Subasinghe et al. 2001). Bacterial disease outbreaks can also lead to temporary or full suspension of stocking into wild fisheries (Piper et al. 1982), potentially impacting both populations of conservation concern and recreational fisheries.

One of the most prevalent salmonid pathogens in the world is the bacterium *Flavobacterium psychrophilum* that causes bacterial coldwater disease (BCWD). Bacterial coldwater disease is characterized by a white discoloration around the adipose fin at the early stages of the disease that transitions into caudal peduncle lesions, revealing underlying muscles and bones (LaFrentz and Cain 2004; Cipriano and Holt 2005). Epizootics of BCWD typically occur at low water temperatures between 4 and 10° C (Borg 1960), accounting for the name *bacterial coldwater disease,* and outbreaks have been reported in different species of salmonids since 1948 (Borg 1960; Michel et al. 1999). *Flavobacterium psychrophilum* infections typically affect age-0 salmonids but can occur in older individuals (Cipriano and Holt 2005; Nicolas et al. 2008; Barnes and Brown 2011). Hatchery mortality associated with infection can be up to 90% (Nilsen et al. 2011; Barnes and Brown 2011) depending on temperature and developmental stage of the host (Wood 1974; Decostere et al. 2001).

Early experimental research led to irreproducible results (Otis 1984; Holt 1987; Chua 1991; Lorenzen 1994; Rangdale 1995) due to difficulties in culturing *F. psychrophilum*. It was not until the late 1990s that researchers improved methods for culturing *F. psychrophilum* so that viability could be maintained after preservation (Daskalov et al. 1999; Michel et al. 1999) allowing for standardization of experimental bacterial exposures within individual laboratories (Garcia et al. 2000; Leeds et al. 2010). The methods reported for challenge experiments vary between laboratories which complicates interpretation among published works and implementation of additional experiments.

Variation among experimental protocols, such as the amount of time bacteria are cultured, the *F. psychrophilum* isolate used, and fish exposure methods, may explain observed differences among challenge experiments. For example, typical bacterial culture times range between 18-hours and 96-hours (Madsen and Dalsgaard 1999; Garcia et al. 2000; Aoki et al. 2005; LaFrentz et al. 2014; Bruce et al. 2020), and, importantly, differences in mortality have been shown to be dependent on culture time (Aoki et al. 2005). Variation in pathogenicity of the different isolates has also been reported worldwide and may explain differences in mortality among studies (Siekoula-Nguedia et al. 2012; Apablaza et al. 2013; Strepparava et al. 2013; Van Vliet et al. 2016). Finally, the method used to expose fish to the bacterium may also play a role in mortality differences observed among studies. Comparisons between exposure methods illustrate that intramuscular injections resulted in higher fish mortality than intraperitoneal injections (Garcia et al. 2000).

Here, I review of the *F. psychrophilum* literature to investigate factors affecting variation in mortality in susceptible salmonids during experimental exposures to *F. psychrophilum*. In particular, I examined how bacterial dose, culture time, exposure method, bacterial isolate, and mass of the fish impacted mortality. In addition, I used Bayesian analytical methods to compare how different protocols affect mortality due to *F. psychrophilum* exposure. Understanding how differences in exposure protocol affect exposure outcomes, like mortality, is essential to advance our knowledge and management of BCWD and the health of cultured salmonids around the world.

METHODS

Meta-data collection

I identified studies published in peer reviewed journals utilizing Web of Science, Academic Search Premier, and Google Scholar using combinations of search terms that are associated with BCWD and *F. psychrophilum* research (Table 1.1): *Flavobacterium psychrophilum*, bacterial coldwater disease, Rainbow Trout Fry Syndrome, challenge, experiment, infection, exposure (e.g., *F. psychrophilum* OR bacterial coldwater disease OR Rainbow Trout Fry Syndrome AND infection AND exposure AND challenge AND experiment). I also identified studies by searching through literature cited sections of papers obtained during the initial search. I only included studies that used Rainbow Trout *Oncorhynchus mykiss* as the study species and reported the *F. psychrophilum* isolate used, mortality (number of dead fish as a response), number of hours the bacterium was cultured, infection/exposure method, bacterial dosage used, weight of fish at the time of infection, and cumulative percent mortality or the number of fish dead out of the total number of fish infected. I extracted data manually from the text, tables, and figures, taking only information pertaining to susceptible (able to contract BCWD) and naïve (never exposed to *F. psychrophilum*) fish within *F. psychrophilum* experiments. Each data point in my analysis consists of the dependent variable, mortality, and associated covariates affecting mortality including dose used to expose the fish (mock exposure controls represented by zero dose), weight of the fish, hours the bacteria were cultured (mock exposure controls represented by zero hours), exposure method (list those here), bacterial isolate, the interaction between dose and fish weight (dose*weight), and the interaction between dose and the exposure method (dose*exposure method).

Meta-Regression

Prior to statistical modeling of mortality, all covariates were examined for linearity/correlation. Each non-numerical categorical variable (e.g., bacterial isolate) was transformed into an indicator variable, represented by a zero or one. Due to large differences in units and scale for each predictor, I standardized all covariate values prior to analysis by subtracting the mean of the covariate group and

dividing by two standard deviations (Gelman and Hill 2007; Table 1.2). Standardization of the covariate values allows for comparison of the covariate effects.

Mortality, measured as the number of dead fish ($y_{i,j}$), was analyzed using a hierarchical Bayesian binomial beta regression model (Appendix 1.1)

$$\mathrm{g}(S_{0,j}, \boldsymbol{S}, x_{i,j}) = \text{inverse logit}(S_{0,j} + S_1 x_{i,j} + \cdots + S_{24} x_{i,j})$$

$$\begin{aligned}[\, \boldsymbol{p}, \boldsymbol{S}, \boldsymbol{S}_0, \sigma^2, \mu_\xi, \sigma_\xi \mid \boldsymbol{y}] \propto \textstyle\prod_{i=1}^{n} \prod_{j=1}^{J} \text{ binomial}(y_{i,j} \mid p_{i,j}) \\ \times \text{beta}(p_{i,j} \mid g(S_j, \boldsymbol{S}, x_{i,j}), \sigma^2) \\ \times \text{normal}(S_{0,j} \mid \mu_\xi, \sigma_\xi) \\ \times \text{normal}(\boldsymbol{S} \mid 0, 1.96) \\ \times \text{uniform}(\sigma^2 \mid 0, 100) \\ \times \text{normalo}(\mu_\xi \mid 0, 1.96) \\ \times \text{uniform}(\sigma_\xi \mid 0, 100)\end{aligned}$$

using Just Another Gibbs Sampler (JAGS) within program R (version 4.0.3). The *i* represents observation, *j* represents manuscript, $p_{i,j}$ represents the probability of mortality, $S_{0,j}$ represents the intercept that varies with manuscript, and $S_{1,..,24}$ represents regression coeffects associated with each covariate. The use of a binomial beta mixture distribution allows for added variation not accounted for with a traditional binomial regression model. Vague prior information was used for each regression coefficient parameter (S) associated with each covariate and the mean of intercepts (μ_ξ) with the means equal to zero and the variance equal to 1.96 from a normal distribution. The variance of 1.96 was used due to the logit link function within the model (Hobbs and Hooten 2015). Vague prior information for variances (σ and σ_ξ) was from a uniform distribution ranging from zero to one hundred.

Posterior inference for model parameters and derived quantities were based on two chains of 550,000 Markov chain Monte Carlo (MCMC) samples following convergence after a burn in period of 20% of the total iterations. Convergence for model parameters was determined by visual inspection of mixed trace plots and the use of Gelman and Rubin diagnostic (Gelman and Rubin 1992). Gelman and Rubin diagnostic values less than 1.1 indicate model convergence (Hobbs and Hooten 2015). Inferences about mortality were evaluated by examining the posterior distribution for each covariate allowing for a

calculated mean and 95% credible interval for each factor. Lack of fit was assessed using posterior predictive checks, or Bayesian p-values, where values < 0.1 and > 0.9 indicate lack of fit (Hobbs and Hooten 2015). I present the multiplicative change in odds of mortality ($e^{\text{regression coefficient}}$), mean effect size, and the associated 95% credible intervals for each covariate to illustrate their impact on fish mortality.

RESULTS

Meta-data

Using my search criteria, 604 manuscripts met one or more criterion and 22 met all search criteria. All 22 studies from which I took data were published since 1999 (Table 1.3). The 22 manuscripts accounted for 132 data points that were used in the analysis. Of the 132 total data points, 41 data points were sham controls (injection exposure or immersion exposure with no bacteria; dose and culture time equal to zero) and 91 were bacterial exposures. Fish weights ranged between 0.3 g and 300 g. Eleven different bacterial isolates were identified (Table 1.3). Bacterial doses ranged from 7.00×10^2 colony forming units per mL (CFU/mL) to 8.90×10^8 CFU/mL. Four methods of mock exposures and bacterial exposures were documented, subcutaneous injection (N = 42 data points), intraperitoneal injection (N = 60), intramuscular injection (N = 12) and bath immersion exposure (N = 18). The duration the bacterium was cultured ranged between 18-hours and 96-hours (Table 3).

Meta-Regression

Visual inspection of trace plots and all Gelman and Rubin diagnostic values equaling 1.0 for all model parameters indicated that the two MCMC chains mixed and converged to the target distributions. The Bayesian p-value for the expected value of mortality was 0.63 and the Bayesian p-value for the standard deviation for the expected value of mortality was 0.28 indicating model fit. The standard deviation of the mortality values among the data points was 0.04. Slopes for each manuscript ranged between -1.19 and 0.061 (Figure 1.1). Regression coefficients all ranged between -1.15 and 0.99 (Table 1.4; Figure 1.2). Standard deviation of the regression coefficients ranged between 0.18 and 0.84, with the coefficient associated with dose having the largest variation (Table 1.4). The probability of mortality ranged between 0.49% and 97.27% across all data points.

Of the 24 regression slope coefficients from the meta-regression that were included in the analysis, five coefficients showed no effect on mortality with similar probabilities of the effect being in the positive or negative direction (including dose, hours, isolate S21, subcutaneous injections, and dose*bath; Figure 1.2; Table 1.4). Six coefficients showed a slight effect on mortality with probabilities showing either an increase or decrease in mortality between 60% and 70% (weight, isolate JIP 02-97, intraperitoneal injection, intramuscular injection, dose*subcutaneous injection, and dose* intramuscular injection; Figure 1.2; Table 1.4). Seven coefficients showed an increase in mortality with the probability of being in the positive direction greater than 70% (dose*weight, isolate CSF259-93, isolate 950106-1/1, isolate AVU-1T/07, isolate 99/1A, isolate 900406-1/3, and dose*intraperitoneal injection; Figure 1.2; Table 1.4). Seven coefficients showed a decrease in mortality with the probability of being in the negative direction greater than 70% (intercept, isolate NCIMB1947, isolate Dubois, isolate 99/10A, isolate FPG-101, control treatment, and bath exposure; Figure 1.2; Table 1.4).

Only four regression coefficients had 95% credible intervals that did not overlap zero (intercept, isolate 900406-1/3, isolate 99/1A, and Dose*Intraperitoneal; Figure 1.2). The multiplicative change in the odds of mortality based on each covariate ranged between 0.32 and 2.68 (Table 1.4). Of the four exposure types, bath immersion exposures showed the lowest multiplicative change in the odds (Table 1.4), indicating that bath exposures produce less mortality than other exposure types. Controls had the largest mean negative effect within the analysis, indicating that this group had the lowest reported mortality of the exposure types. The interaction between dose and intraperitoneal injection (Dose*Intraperitoneal) was the only exposure type effect that had 95% credible intervals not overlapping zero indicating an increase in mortality when intraperitoneal injection was used as the exposure method. Dose*Intraperitoneal also had the least variability, with the smallest 95% credible intervals, of the effects that involved exposure type (Figure 1.2).

Of the eleven *F. psychrophilum* isolates included within the analysis, six isolates showed negative effects and five isolates showed positive effects on mortality (Figure 1.1). Nine isolates had 95% credible intervals that overlapped with zero, and nine out of eleven isolates had 95% credible intervals that

overlapped with each other. Isolate S21 was the only isolate that had relatively similar probabilities of having both positive and negative effects on mortality (49% probability of being negative and 51% probability of being positive). All other isolates had a 60% or greater probability of having either a positive or negative effect on mortality. Despite some overlap of the 95% credible intervals (e.g., 900406-1/3 versus 99/1A or NCIMB1947 versus JIP 02-97), I found some evidence that several bacterial isolates produce higher mortality than others (FPG-101 versus 900406-1/3, FPG-101 versus 99/1A, 900406-1/3 versus 99/10A, 99/1A versus 99/10A, 900406-1/3 versus JIP 02-97, 99/1A versus JIP 02-97, 900406-1/3 versus NCIMB1947, and 99/1A versus NCIMB1947; Figure 1.2). The largest overlap among the comparisons is 15.28% (900406-1/3 versus JIP 02-97), indicating that 84.72% of the time there is a difference between these two isolates.

DISCUSSION

My meta-analysis of the published literature for experimental infections with *F. psychrophilum* on susceptible/naïve Rainbow Trout indicated that there is variation among individual labs, as well as variation among the isolates and exposure methods used in challenge experiments. The analysis also showed differences in mortality due to dose, fish weight at exposure, bacterial isolate, and exposure type.

This is the first quantitative meta-analysis of the variation seen among studies concerning *F. psychrophilum* exposure mortality. Evaluating variation in the individual covariates was not an *a priori* goal; however, it appears that standardization is occurring within labs but not among labs. My meta-analysis shows that mortality from *F. psychrophilum* is variable among studies published since 1999, despite advances in culture methods and individual laboratory standardization of experimental methods. The difficulty in comparing experimental infection results within the literature is likely due to varied methods utilized from one laboratory to another, and this is evident when looking at the effects of the individual covariates showing broad variation; it is unclear as to what is truly driving the variation of the effect for individual covariates. One possibility is that there is variation not being accounted for within the model, such as Rainbow Trout strain. Differences in genetics within Rainbow Trout result in differences in mortality for the same dose and exposure method (chapters within this dissertation). If

reducing variation in mortality were warranted, one possible way would be to choose the bacterial isolate or exposure method that should result in the smallest variation based on the research goals.

A surprising result of the meta-analysis was that there was a positive effect of fish weight on the number of mortalities based on the dose*weight interaction, suggesting that, for the same dose, weight increases the odds of mortality (a multiplicative change of 1.49 when all other covariate values are held constant). The effect of dose*weight contradicts other observations in reviews and challenge experiments, which generally show that smaller age-0 fish have higher mortality than larger fish (Madsen and Dalsgaard 1999; Decostere et al. 2001; LaFrentz and Cain 2004). One potential reason for the difference between my results and others could be the limitation that published studies are focused on characterizing BCWD infection in relatively small fish. The maximum weight used within this meta-analysis was 300 g (representing only two data points). However, the rest of the weight data ranged between 0.33 g to 28 g, with the majority of the weights being below 10 g. The data reported in the literature does not represent the potential size range of Rainbow Trout, which may bias the effect that weight has on mortality associated with *F. psychrophilum* infection. Another reason for the discrepancy could be a non-linear effect of weight in which mortality initially increases for age-0 fish and declines after fish reach a larger size. Investigation of non-linear effects would require a broader range of fish weights than are currently reported in the literature. Future experiments conducted with a broad range of weights are needed to assess the effect of size and age on mortality associated with BCWD.

My results indicated that exposure to *F. psychrophilum* by injection produces more mortality than by bath immersion. One potential reason for higher mortality is that injection exposures allow the bacteria to bypass the external immune responses of the fish compared to bath immersion exposure. However, bath exposure was unevenly represented in the reported literature where only 12 of the 132 cases of mortality involved bath exposure. It appears that intraperitoneal injection leads to more mortality compared to intramuscular or subcutaneous injection based on the dose*intraperitoneal interaction. Given the smaller variation in mortality associated with intraperitoneal injection shown in my analysis, intraperitoneal injection may be preferable. Providing proper administration and protocols, injections not

damaging organs and the incorporation of injection controls, intraperitoneal injection should provide more consistent mortality in *F. psychrophilum* challenge experiments given that the goal is to assess bacteria-caused mortality, not mortality from the exposure process itself; more consistent protocols and results may lead to easier comparison between experiments involving *F. psychrophilum* exposure.

My analysis suggests that bacterial isolates differ in their effect on mortality, with some producing higher mortality compared to others. The benefit of using a Bayesian approach is that it allows comparison of distributions, even if they overlap, and calculation of the probability of an effect being positive or negative. Some isolates, such as FPG-101 and 99/10A as well as Dubois and S21 have 100% overlap, indicating no difference in mortality between the isolates. Despite the overlap observed for some isolates there are several comparisons between isolates, with 900406-1/3 and JIP 02-97 being the largest, that show an 86.47% probability of producing differences in mortality. Comparisons between other isolates also indicate a relatively high probability of differing in their effect on mortality, while some isolates do not seem to differ in their effects on fish mortality. Differences in virulence and mortality between *F. psychrophilum* isolates have been shown within the literature (Siekoula-Nguedia et al. 2012; Apablaza et al. 2013; Strepparava et al. 2013; Van Vliet et al. 2016; Jarau et al. 2018). Additionally, studies show that *F. psychrophilum* has high genetic heterogeneity (Siekoula-Nguedia et al. 2012; Avendano-Herrera et al. 2014; Nilsen et al. 2014, Van Vliet et al. 2016), genetic variation among isolates may be connected to variation in virulence (Van Vliet et al. 2016) which may explain the variation in mortality attributed to different isolates. It also seems likely that unique isolates would affect Rainbow Trout populations differentially. Rainbow Trout strain was not included in my analysis because it was not commonly reported but may explain some of the variability in mortality that is seen among isolates. Rainbow Trout populations that have not been frequently exposed to a specific isolate may show higher mortality compared to Rainbow Trout that have been exposed to that same isolate for a long time. Similar disease dynamics have been observed with variable susceptibility to whirling disease among Rainbow Trout strains (Hedrick et al. 2003; Fetherman et al. 2012). Further research is required to

describe how Rainbow Trout strain affects mortality based on the bacterial isolate exposure to determine whether Rainbow Trout strain needs to be accounted for in *F. psychrophilum* challenge experiments.

Culture time had no effect on mortality and was unevenly represented in the literature. A total of 82 data points represented culture times of 48 hours or greater (44, 48-hours; 2, 66-hours; 33, 72-hours; 3, 96-hours). Only 20 data points represented culture times ranging between 18 and 36 hours (4, 18-hours; 5, 24-hours; 11, 36-hours) with the remaining 30 data points representing control exposures with zero culture time. One explanation for higher culture time reported in the literature is the potential for maximizing numbers of bacteria harvested and an overall higher concentration of bacteria for experimental use. However, as culture time increases, resources in the culture media decrease, leading to a combination of bacteria that are actively replicating and inactive bacteria that are in a stationary or non-growth phase (Wang et al. 2015). A potential reason for culture time spanning both negative and positive values and showing variable effects on mortality is that bacteria in the stationary growth phase may have lower virulence due to there being fewer actively replicating bacteria. Kondo et al. (2001) suggested that *F. psychrophilum* harvested at the logarithmic phase (the maximum population growth rate of the bacteria) may result in higher virulence. Aoki et al. (2005) showed that bacteria cultured at 18 and 24 hours resulted in greater fish mortality than bacteria cultured at 48 hours. The meta-data suggests that the current *F. psychrophilum* published literature is lacking in experimental infections when *F. psychrophilum* were harvested during their logarithmic growth phase, ranging between 18 and 36 hours of culture time (Michel et al. 1999; Aoki et al. 2005). Bacteria harvested during the logarithmic growth phase may provide more consistent fish mortality rates since the bacteria are all actively replicating.

Bacterial dose is an important aspect to *F. psychrophilum* exposure experiments. Mortality has been shown to be dose dependent (Madsen and Dalsgaard 1999; Garcia et al. 2000; chapter 2 appendix) indicating that dose needs to be accounted for in exposure experiments. The meta-analysis suggested that dose by itself has no effect on mortality and is highly variable. However, the increase in mortality due to dose*weight interaction and dose*intraperitoneal injection interaction indicate that dose does affect mortality due to *F. psychrophilum* exposure. Mortality based on dose is expected to be variable because

of the differences in isolates, exposure method, and different fish weights. Because there is variable mortality based on bacterial dose, pilot experiments (see Chapter 2 appendix) will provide information until standardized procedures are achieved within the laboratory.

My meta-analysis allows for inference on what factors affect mortality by collecting data from across the peer-reviewed literature, in the absence of conducting more experiments, and formally comparing factors using Bayesian statistics. Variation in mortality of *F. psychrophilum* exposure experiments has been observed due to the different isolates and exposure protocols used across laboratories. To advance our understanding of BCWD and its effects on the health of cultured salmonids around the world, it is necessary to establish protocols that will result in comparable outcomes across laboratories, provide the ability to use prior experimental information in future experiments, and to evaluate management options to mitigate *F. psychrophilum* infection. Moving forward, the meta-analysis results provide steppingstones for reducing *F. psychrophilum* mortality variation and, in turn, future results that may be comparable across laboratories.

Table 1.1. The search terms and Boolean terms used for data collection in Web of Science, Academic Search Premier, and Google Scholar.

	Search Terms
1	*Flavobacterium psychrophilum*
2	bacterial coldwater disease
3	Rainbow Trout Fry Syndrome
4	*Flavobacterium psychrophilum* AND challenge
5	bacterial coldwater disease AND challenge
6	Rainbow Trout Fry Syndrome AND challenge
7	*Flavobacterium psychrophilum* AND experiment
8	bacterial coldwater disease AND experiment
9	Rainbow Trout Fry Syndrome AND experiment
10	*Flavobacterium psychrophilum* AND exposure
11	bacterial coldwater disease AND exposure
12	Rainbow Trout Fry Syndrome AND exposure
13	*Flavobacterium psychrophilum* AND infection
14	bacterial coldwater disease AND infection
15	Rainbow Trout Fry Syndrome AND infection
16	*Flavobacterium psychrophilum* AND challenge OR experiment OR exposure OR infection
17	bacterial coldwater disease AND challenge OR experiment OR exposure OR infection
18	Rainbow Trout Fry Syndrome AND challenge OR experiment OR exposure OR infection
19	*Flavobacterium psychrophilum* OR bacterial coldwater disease OR Rainbow Trout Fry Syndrome AND challenge OR experiment OR exposure OR infection

Table 1.2. Values used to standardize covariates used in the meta-analysis of *F. psychrophilum* experiments. Standardization provided a unitless value for each covariate that could be used for comparisons across covariates in the meta-analysis. Covariates examined in the analysis included dose (CFU/mL), weight (g), dose*weight, hours, isolate (CSF259-93 through FPG-101), exposure type (none [Control or mock injection]; subcutaneous, intraperitoneal, or intramuscular injection; bath), and dose*exposure type and were standardized by subtracting the covariate mean and dividing by two standard deviations from each data point.

Covariate Data	Mean	2*(Standard Deviation)
Dose	33531108.96	237498476
Weight	9.4767537313	72.732591605
Dose*Weight	128426577	646708166.8
Hours	41.73134328	54.42421187
Isolate: CSF259-93	0.26119403	0.881867618
Isolate: NCIMB1947	0.059701493	0.475644021
Isolate: S21	0.014925373	0.243418588
Isolate: Dubois	0.02238806	0.296994257
Isolate: 950106-1/1	0.119402985	0.650957704
Isolate: JIP 02-97	0.067164179	0.50249071
Isolate: AVU-1Y/07	0.014925373	0.243418588
Isolate: 99/1A	0.044776119	0.415176139
Isolate: 99/10A	0.044776119	0.415176139
Isolate: 900406-1/3	0.037313433	0.380479825
Isolate: FPG-101	0.014925373	0.243418588
None	0.298507463	0.918640733
Subcutaneous	0.313432836	0.931258682
Intraperitoneal	0.447761194	0.998259061
Intramuscular	0.089552239	0.573221293
Bath	0.134328358	0.684568676
Dose*Subcutaneous	8598694.03	96790480.46
Dose*Intraperitoneal	2552562.687	17310000.86
Dose*Intramuscular	1505971.642	17914526.18
Dose*Bath	20776865.67	221064624.2

Table 1.3. Data used within the meta-analysis showing how many data points were included, *F. psychrophilum* dose range (CFU/mL), exposure type (subcutaneous injection, intramuscular injection, intraperitoneal injection, and bath immersion), isolate used, weight range (g), and number of culture hours used from each author.

Data Points	Dose Range	Exposure Type	Isolate	Weight (g)	Culture Hours	Manuscript	Author
10	$2.0x10^6$ - $3.4x10^8$	Bath	NCIMB1947	1.1 - 6.4	18 - 66	6	Aoki et al. 2005
5	$1.34x10^5$ - $7.8x10^7$	Intramuscular	CSF259-93, S21	1.4 - 11.3	18 - 72	22	Bruce et al. 2020
8	$3.0x10^5$ - $7.0x10^6$	Subcutaneous	CSF259-93	5	72	8	Burbank et al. 2011
2	$1.4x10^7$	Intraperitoneal	950106-1/1	3.5	48	19	Chettri et al. 2018
6	$1.0x10^6$	Intraperitoneal, Intramuscular	Dubois	1 - 300	72	3	Decostere et al. 2001
11	$4.2x10^3$ - $4.2x10^7$	Intraperitoneal	JIP 02-97	4.5	36	2	Garcia et al. 2000
2	$1.2x10^7$	Intraperitoneal	CSF259-93	0.5	72	15	Ghosh et al. 2016
3	$2.0x10^8$ - $5.0x10^8$	Subcutaneous	CSF259-93	2.3	72	10	Glenn et al. 2014
2	$1.0x10^5$ - $1.0x10^7$	Bath	950106-1/1	0.77	24 - 48	9	Henriksen et al. 2013
2	$1.0x10^8$	Bath	AVU-1T/07	12.46, 12.75	24	18	Hoare et al. 2017
12	$1.25x10^4$ - $1.25x10^6$	Subcutaneous	CSF259-93	1.4	72	4	LaFrentz et al. 2003
3	$6.25x10^6$ - $6.25x10^7$	Subcutaneous	CSF259-93	7.5	72	5	LaFrentz et al. 2004
6	$6.25x10^6$ - $6.25x10^7$	Subcutaneous	CSF259-93	4.6, 15	48	7	LaFrentz et al. 2008
6	$5.0x10^7$ - $1.20x10^8$	Subcutaneous	CSF259-93	5, 10	96	11	LaFrentz et al. 2014
4	$7.9x10^8$ - $8.9x10^8$	Bath	CSF259-93	0.3, 1.5	48	12	Long et al. 2014
1	$4.7x10^7$	Intramuscular	CSF259-93	3.5	72	20	Ma et al. 2018
2	$1.0x10^6$	Subcutaneous	CSF259-93	3.5	72	21	Ma et al. 2019
31	700 - $4.0x10^7$	Intraperitoneal	950106-1/1, 99/1A, 99/10A, 900406-1/3	1.0 - 28	48	1	Madsen and Dalsgaard 1999
4	$3.03x10^7$	Intraperitoneal	FPG-101	7.5	72	16	Ryerse et al. 2016
2	$1.03x10^7$	Subcutaneous	CSF259-93	4	72	14	Schubiger et al. 2015
4	$2.29x10^7$ - $3.0x10^7$	Intraperitoneal	CSF259-93	3.08, 4.27	72	17	Sudheesh and Cain 2016
6	$3.6x10^6$ - $6.3x10^7$	Intraperitoneal	CSF259-93	6.8 – 9.5	72	13	Wagner and Oplinger 2014

Table 1.4. Posterior regression coefficients (S) from standardized data with mean values, standard deviation values, the represented multiplicative change and the probability of the regression coefficient being negative or positive for dose (CFU/mL), weight (g), dose*weight, hours, isolate (CSF259-93 through FPG-101), exposure type (none [Control or mock injection]; subcutaneous, intraperitoneal, or intramuscular injection; bath), and dose*exposure type.

Parameter	Mean	Standard Deviation	Multiplicative Change	[Neg., Pos.]
S_{Dose}	0.093	0.84	1.10	[46%, 54%]
S_{Weight}	-0.060	0.18	0.94	[67%, 33%]
$S_{Dose*Weight}$	0.32	0.33	1.38	[14%, 86%]
S_{Hours}	0.067	0.28	1.070	[41%, 59%]
$S_{CSF259-93}$	0.57	0.62	1.76	[18%, 82%]
$S_{NCIMB1947}$	-0.43	0.40	0.65	[86%, 14%]
S_{S21}	-0.011	0.49	0.99	[49%, 51%]
S_{Dubois}	-0.34	0.30	0.71	[89%, 11%]
$S_{950106-1/1}$	0.46	0.48	1.58	[17%, 83%]
$S_{JIP\ 02-97}$	-0.15	0.45	0.86	[63%, 37%]
$S_{AVU-1T/07}$	0.28	0.37	1.32	[22%, 78%]
$S_{99/1A}$	0.99	0.35	2.68	[0.2%, 99.8%]
$S_{99/10A}$	-0.27	0.40	0.76	[76%, 24%]
$S_{900406-1/3}$	0.89	0.32	2.44	[0.2%, 99.8]
$S_{FPG-101}$	-0.26	0.31	0.77	[82%, 18%]
S_{None}	-1.15	0.63	0.32	[96%, 4%]
$S_{Subcutaneous}$	0.14	0.70	1.14	[42%, 58%]
$S_{Intraperitoneal}$	0.19	0.74	1.21	[40%, 60%]
$S_{Intramuscular}$	0.17	0.48	1.18	[36%, 64%]
S_{Bath}	-0.60	0.56	0.55	[86%, 14%]
$S_{Dose*Subcutaneous}$	0.18	0.41	1.20	[32%, 68%]
$S_{Dose*Intraperitoneal}$	0.69	0.19	1.98	[0.1%, 99.9%]
$S_{Dose*Intramuscular}$	0.23	0.51	1.26	[31%, 68%]
$S_{Dose*Bath}$	-0.044	0.78	0.96	[52%, 48%]

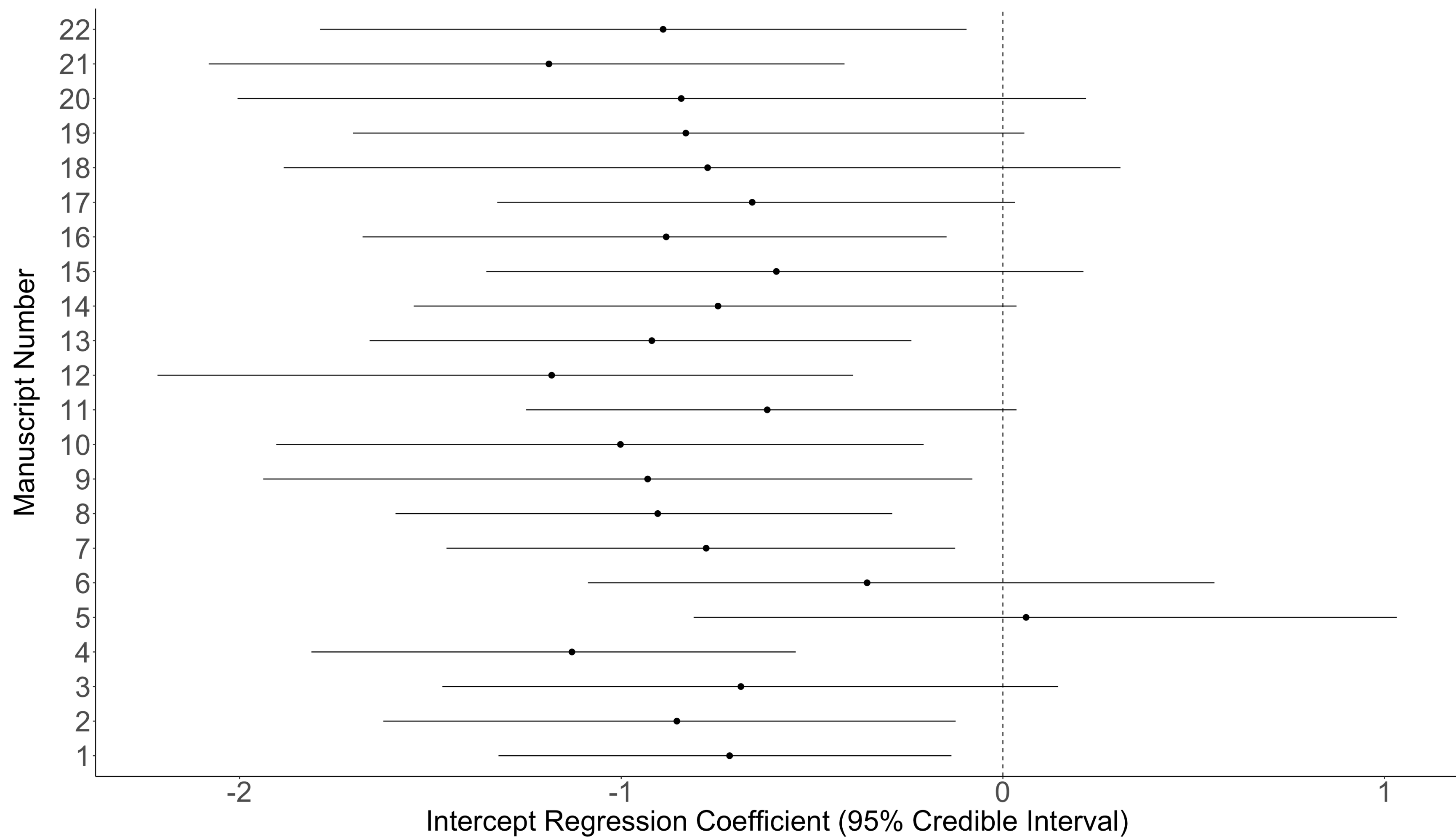

Figure 1.1. Calculated intercepts (x-axis) for each manuscript (y-axis) Madsen and Dalsgaard 1999, Garcia et al. 2000, Decostere 2001, LaFrentz et al. 2003, LaFrentz et al. 2004, Aoki et al. 2005, LaFrentz et al. 2008, Burbank et al. 2011, Henriksen et al. 2013, Glenn et al. 2014, LaFrentz et al. 2014, Long et al. 2014, Wagner and Oplinger 2014, Schubiger et al. 2015, Ghosh et al. 2016, Ryerse et al. 2016, Sudheesh and Cain 2016, Hoare et al. 2017, Chettri et al. 2018, Ma et al. 2018, Ma et al. 2019, and Bruce et al. 2020. Black dots represent the mean and the horizontal lines represent the 95% credible intervals. Vertical dotted black line denotes zero.

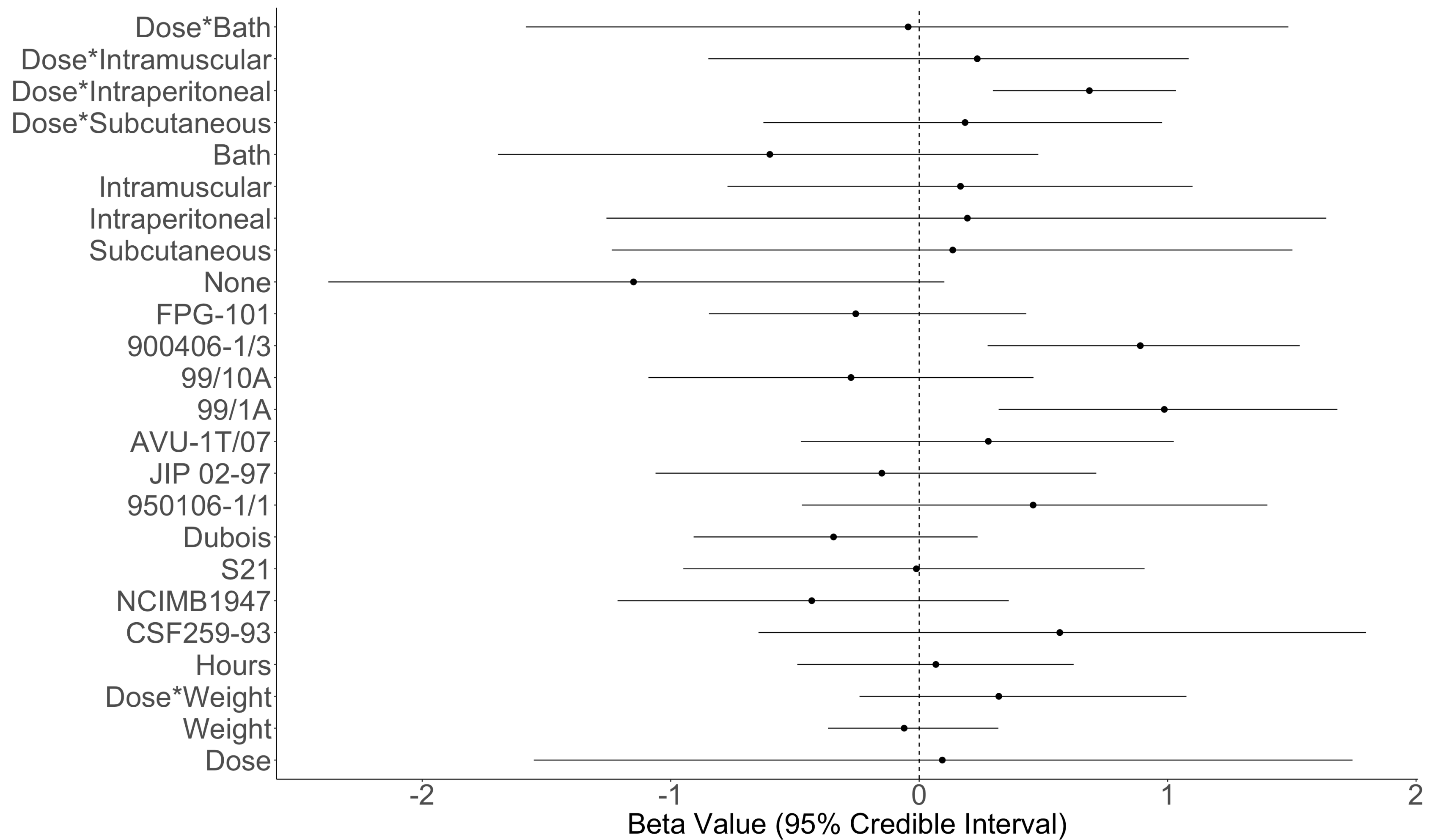

Figure 1.2. Calculated regression coefficients (x-axis) for each covariate (y-axis) dose (CFU/mL), weight (g), dose*weight, hours, isolate (CSF259-93 through FPG-101), exposure type (none [Control or mock injection]; subcutaneous, intraperitoneal, or intramuscular injection; bath), and dose*exposure type. Black dots represent the mean and the horizontal lines represent the 95% credible intervals. Vertical dotted black line denotes zero.

LITERATURE CITED

Aoki, M., M. Kondo, K. Kawai, and S. Oshima. 2005. Experimental bath infection with *Flavobacterium psychrophilum*, inducing typical signs of Rainbow Trout *Oncorhynchus mykiss* fry syndrome. Diseases of Aquatic Organisms 67(1-2):73-79.

Apablaza, P., A. D. Loland, O. J. Brevik, P. Ilardi, J. Battaglia, and A. Nylund. 2013. Genetic variation among *Flavobacterium psychrophilum* isolates from wild and farmed salmonids in Norway and Chile. Journal of Applied Microbiology 114:943-946.

Avendano-Herrera, R., A. Houel, R. Irgang, J. F. Bernardet, M. Godoy, P. Nicolas, and E. Duchaud. 2014. Introduction, expansion and coexistence of epidemic *Flavobacterium psychrophilum* lineages in Chilean fish farms. Journal of Veterinary Microbiology 170:298-306.

Barnes, M. E., and M. L. Brown. 2011. A review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment. The Open Fish Science Journal 4:1-9.

Berger, L., R. Speare, and P. Daszak. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America 95:9031–9036.

Borg, A. F. 1960. Studies on myxobacteria associated with diseases in salmonid fishes. Journal of Wildlife Disease 8:1-85.

Bruce, T. J., J. Ma, C. Knupp, T. P. Loch, M. Faisal, and K. D. Cain. 2020. Cross-protection of a live-attenuated *Flavobacterium psychrophilum* immersion vaccine against novel *Flavobacterium* spp. and *Chryseobacterium* spp. strains. Journal of Fish Diseases 43:915-928.

Burbank, D. R., D. H. Shah, S. E. LaPatra, G. Fornshell, and K. D. Cain. 2011. Enhanced resistance to coldwater disease following feeding of probiotic bacterial strains to Rainbow Trout (*Oncorhynchus mykiss*). Aquaculture 321:185-190.

Chettri, J. K., A. Al-Jubury, I. Dalsgaard, P. M. H. Heegaard, and K. Buchmann. 2018. Experimental anal infection of Rainbow Trout with *Flavobacterium psychrophilum*: A novel challenge model. Journal of Fish Diseases 41:1917-1919.

Chua, F. H. C. 1991. A study of the Rainbow Trout fry syndrome. Master's thesis. University of Stirling.

Cipriano, R. C., and R. A. Holt. 2005. *Flavobacterium psychrophilum*, cause of bacterial cold-water disease and rainbow trout fry syndrome. United States Department of Interior, U.S. Geological Service, National Fish Health Research Laboratory. Fish Disease Leaflet No. 86.

Daskalov, H., D. A. Austin, and B. Austin. 1999. An improved growth medium for *Flavobacterium psychrophilum*. Letters in Applied Microbiology 28:297-299.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5(6):735-748.

Decostere, A., E. D'Haese, M. Lammens, H. Nelis, and F. Haesebrouck. 2001. *In vivo* study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes. Journal of Fish Diseases 24:481-487.

Fetherman, E. R., D. L. Winkelman, M. R. Baerwald, and G. J. Schisler. 2014. Survival and reproduction of *Myxobolus cerebralis*-resistant Rainbow Trout introduced to the Colorado River and increased resistance of age-0 progeny. PLoS ONE. 9(5):e96954.

Garcia, C., F. Pozet, and C. Michel. 2000. Standardization of experimental infection with *Flavobacterium psychrophilum*, the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. Diseases of Aquatic Organisms 42:191-197.

Gelman, A., and D. B. Rubin. 1992. Inference from iterative simulation using multiple sequences. Statistical Science 7:457-472.

Gelman, A., and J. Hill. 2007. Data analysis using regression and multilevel/hierarchical models. Cambridge University Press. New York.

Ghosh, B., K. D. Cain, B. F. Nowak, and A. R. Bridle. 2016. Microencapsulation of a putative probiotic *Enterobacter* species, C6-6, to protect Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), against bacterial coldwater disease. Journal of Fish Diseases 39:1-11.

Glenn, R. A., A. L. Gannam, and S. E. LaPatra. 2014. The lack of effectiveness of roemary oil on fish feed in controlling bacterial cold-water disease in Rainbow Trout. North American Journal of Aquaculture 76:359-363.

Hedrick, R. P., T. S. McDowell, G. D. Marty, G. T. Fosgate, K. Mukkatria, K. Mykelbust, and M. El-Matbouli. 2003. Susceptibility of two strains of Rainbow Trout (one with suspected resistance to whirling disease) to *Myxobolus cerebralis* infection. Disease of Aquatic Organisms 55:37-44.

Henriksen, M. M. M., L. Madsen, and I. Dalsgaard. 2013. Effect of hydrogen peroxide on immersion challenge of Rainbow Trout fry with *Flavobacterium psychrophilum*. PLoS ONE 8(4):e62590.

Hobbs, N. T., and M. B. Hooten. 2015. Bayesian models a statistical primer for ecologists. Princeton University Press, Princeton, New Jersey.

Holt, R. A. 1987. *Cytophaga psychrophile*, the causative agent of bacterial cold-water disease in salmonid fishes. Doctoral dissertation. Oregon State University, Corvallis.

Hoare, R., T. P. H. Ngo, K. L. Bartie, and A. Adams. 2017. Efficacy of a polyvalent immersion vaccine against *Flavobacterium psychrophilum* and evaluation of immune response to vaccination in Rainbow Trout fry (*Oncorhynchus mykiss* L.). Veterinary Research 48(43):1-13.

Jarau, M., A. Di Natale, P. E. Huber, J. I. MacInnes, and J. S. Lumsden. 2018. Virulence of *Flavobacterium psychrophilum* isolates in Rainbow Trout *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 41:1205-1514.

Klesius, P. H., and J. W. Pridgeon. 2011. Live attenuated bacterial vaccines in aquaculture. *In*: Proceedings of the 9th International Symposium on Tilapia in Aquaculture p. 18-26.

Kondo, M., K. Kawai, K. Yagyu, K. Nakayama, K. Kurohara, and S. Oshima. 2001. Changes in the cell structure of *Flavobacterium psychrophilum* with length of culture. Microbiology and Immunology 45(12):813-818.

Kuo, L., and B. Mallick. 1998. Variable selection for regression models. Sankhya: The Indian Journal of Statistics, Series B 60(1):65-81.

LaFrentz, B. R., S. E. LaPatra, G. R. Jones, and K. D. Cain. 2003. Passive immunization of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and Rainbow Trout fry syndrome. Journal of Fish Diseases 26:377-384.

LaFrentz, B. R., and K. D. Cain. 2004. Bacterial coldwater disease. An Extension Bulletin for the Western Regional Aquaculture Center. University of Idaho, Moscow.

LaFrentz, B. R., S. E. LaPatra, G. R. Jones, and K. D. Cain. 2004. Protective immunity in Rainbow Trout *Oncorhynchus mykiss* following immunization with distinct molecular mass fractions isolated from *Flavobacterium psychrophilum*. Diseases of Aquatic Organisms 59:17-26.

LaFrentz, B. R., S. E. LaPatra, D. R. Call, and K. D. Cain. 2008. Isolation of rifampicin resistant *Flavobacterium psychrophilum* strains and their potential as live attenuated vaccine candidates. Vaccine 26:5582-5589.

LaFrentz, B. R., S. E. LaPatra, D. R. Call, and K. D. Cain. 2014. Immunization of Rainbow Trout *Oncorhynchus mykiss* (Walbaum) with a crude lipopolysaccharide extract from *Flavobacterium psychrophilum*. Aquaculture Research 45:476-483.

Leeds, T. D., J. T. Silverstein, G. M. Weber, R. L. Vallejo, Y. Palti, C. E. Rexroad III, J. Evenhuis, S. Hadidi, T. J. Welch, and G. D. Wiens. 2010. Response to selection for bacterial cold water disease resistance in Rainbow Trout. Journal of Animal Science 88:1936-1946.

Long, A. T., T. R. Fehringer, B. R. LaFrentz, D. R. Call, and K. D. Cain. 2014. Development of a waterborne challenge model for *Flavobacterium psychrophilum*. FEMS Microbiology Letters 359:154-160.

Lorenzen, E. 1994. Studies of *Flexibacter psychrophilus* in relation to Rainbow Trout fry syndrome (RTFS). PhD dissertation., Royal Veterinary and Agricultural University, Copenhagen.

Ma, J., T. J. Bruce, P. S. Sudheesh, C. Knupp, T. P. Loch, M. Faisal, and K. D. Cain. 2018. Assessment of cross-protection to heterologous strains of *Flavobacterium psychrophilum* following vaccination with a live-attenuated coldwater disease immersion vaccine. Journal of Fish Diseases 42:75-84.

Ma, J., T. J. Bruce, L. P. Oliver, and K. D. Cain. 2019. Co-infection of Rainbow Trout (*Oncorhynchus mykiss*) with infectious hematopoietic necrosis virus and *Flavobacterium psychrophilum*. Journal of Fish Diseases 00:1-12.

Madsen, L., and I. Dalsgaard. 1999. Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in Rainbow Trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 36:169-176.

Michel, C., D. Antonio, and R. P. Hedrick. 1999. Production of viable cultures of *Flavobacterium psychrophilum*: approach and control. Research in Microbiology 150:351-358.

Muths, E., P. S. Corn, A. P. Pessier, and D. E. Green. 2003. Evidence for disease-related amphibian decline in Colorado. Biological Conservation 110:357–365.

Nicolas, P., S. Mondot, F. Achaz, C. Couchenot, J. Bernardet, and E. Duchaud. 2008. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Applied and Environmental Microbiology 74:3702-3709.

Nilsen, H., A. B. Olsen, O. Vaagnes, H. Hellberg, K. Bottolfsen, H. Skjelstad, and D. J. Coloquhoun. 2011. Systemic *Flavobacterium psychrophilum* infection in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. Journal of Fish Diseases 34:403-408.

Nilsen, H., K. Dundell, E. Duchaud, P. Nicolas, I. Dalsgaard, L. Madsen, A. Aspan, E. Jansson, D. J. Colquhoun, and T. Wiklund. 2014. Multilocus sequence typing identifies epidemic clones of *Flavobacterium psychrophilum* in Nordic countries. Applied and Environmental Microbiology 80:2728-2736.

Otis, E. J. 1984. Lesions of cold-water disease in steelhead trout (*Salmo gairdneri*): the role of *Cytophaga psychrophile* extracellular products. Master's thesis. University of Rhode Island, Kingston.

Piper, R. G., I. B. McElwain, L. E. Orme, J. P. McCaren, L. G. Fowler, and J. R. Leonard. 1982. Fish Hatchery Management (No. 2175). U.S. Fish and Wildlife Service, Washington, D.C.

Pridgeon, J. W., and P. H. Klesius. 2012. Major bacterial diseases in aquaculture and their vaccine development. CAB Reviews 7:1-16.

Rangdale, R. E. 1995. Studies on Rainbow Trout fry syndrome (RTFS). PhD dissertation., University of Stirling.

Robinson, R. A., B. Lawson, M. P. Toms, K. M. Peck, J. K. Kirkwood, J. Chantrey, I. R. Clatworthy, A. D. Evans, L. A. Hughs, O. C. Hutchinson, S. K. John, T. W. Pennycott, M. W. Perkins, P. S. Rowley, V. R. Simpson, K. M. Tyler, and A. A. Cunningham. 2010. Emerging infectious disease leads to rapid population declines of common British birds. PLoS ONE. 5(8):e12215.

Ryerse, I. A., J. M. Hooft, D. P. Bureau, M. A. Hayes, and J. S. Lumsden. 2016. Diets containing corn naturally contaminated with deoxynivalenol reduces the susceptibility of Rainbow Trout (*Oncorhynchus mykiss*) to experimental *Flavobacterium psychrophilum* infection. Aquaculture Research 47:787-796.

Schubiger, C. B., L. H. Orfe, P. S. Sudheesh, K. D. Cain, D. H. Shah, and D. R. Call. 2015. Entericidin is required for probiotic treatment (*Enterobacter* sp. Strain C6-6) to protect trout from cold-water disease challenge. Applied and Environmental Microbiology 81(2):658-665.

Siekoula-Nguedia, C., G. Blanc, E. Duchaud, and S. Calvez. 2012. Genetic diversity of *Flavobacterium psychrophilum* isolated from Rainbow Trout in France: predominance of a clonal complex. Veterinary Microbiology 161:169-178.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A. D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125-134.

Strepparava, N., P. Nicolas, T. Wahli, H. Segner, and O. Petrini. 2013. Molecular epidemiology of *Flavobacterium psychrophilum* from Swiss fish farms. Diseases of Aquatic Organisms 105:203-210.

Subasinghe, R. P., M. G. Bondad-Reantaso, and S. E. McGladdery. 2001. Aquaculture development, health and wealth. *In*: R. P. Subasinghe, P. Bueno, M. J. Phillips, C. Hough, S. E. McGladdery, J. R. Arthur, editors. Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium Bangkok: NACA and FAO p. 167–91. Accessed at: http://www.fao.org/docrep/003/ab412e/ab412e09.htm

Sudheesh, P. S., and K D. Cain. 2016. Optimization of efficacy of a live attenuated *Flavobacterium psychrophilum* immersion vaccine. Fish and Shellfish Immunology 56:169-180.

Van Vliet, D., G. D. Wiens, T. P. Loch, P. Nicolas, and M. Faisal. 2016. Genetic diversity of *Flavobacterium psychrophilum* isolates from three *Oncorhynchus* spp. in the United States, as revealed by multilocus sequence typing. Applied and Environmental Microbiology 82(11):3246-3255.

Vincent, E. R. 1996. Whirling disease and wild trout: the Montana experience. Fisheries 21(6):32-34.

Wagner, E. J., and R. W. Oplinger. 2014. Comparison of the susceptibility of four Rainbow Trout strains to cold-water disease. Journal of Aquatic Animal Health 26:160-167.

Wang, L., D. Fan, W. Chen, and E. M. Terentjev. 2015. Bacterial growth, detachment and cell size control on polyethylene terephthalate surfaces. Scientific Reports 5(1):1-11.

Wood, J. W. 1974. Diseases of Pacific Salmon: their prevention and treatment, 2nd edition. Washington Department of Fisheries, Olympia.

CHAPTER 2: DUAL RESISTANCE TO FLAVOBACTERIUM PSYCHROPHILUM AND MYXOBOLUS CEREBRALIS IN RAINBOW TROUT

INTRODUCTION

Global fish production has increased steadily since the 1950s, with the amount of wild captured fish reaching a plateau in early 2000. The biomass of fish produced by aquaculture showed a steady increase after 2000 (FOA 2020). In recent years, aquaculture production accounts for over 60% of the global fish production value, indicating aquaculture is an important resource worldwide. Within the United States, 169 million tons of trout are produced for food and restoration/conservation practices and are worth more than $230 million US dollars (USDA 2020). Losses within the United States trout production average roughly 30 million trout each year, with disease being the reason for 90% of the trout losses (USDA 2016, 2017, 2018, 2019, 2020).

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD), is found in cultured and wild fishes worldwide and causes significant infection in captive salmonid populations (LaFrentz and Cain 2004; Starliper 2011). Mortality associated with infections can be as high as 90% (Barnes and Brown 2011; Nilsen et al. 2011) depending on water temperature and developmental stage of the host (Wood 1974; Decostere et al. 2001). Due to mortalities associated with BCWD, outbreaks can result in massive economic losses to producers of salmon and Rainbow Trout *Oncorhynchus mykiss* (Antaya 2008). As a result, BCWD is considered one of the most important hatchery diseases in the world (Michel et al. 1999). Infections typically affect age-0 salmonids (Cipriano and Holt 2005; Nicolas et al. 2008) but can also affect larger and older fish (LaFrentz and Cain 2004). Infected fish show a broad range of clinical disease signs such as discoloration of the adipose fin, lesions, spiral swimming behavior, “blacktail”, spinal deformities, and pale or necrotic gills (Davis 1946; Borg 1960; Kent et al. 1989; Ostland et al. 1997; Martinez et al. 2004).

Antibiotics are the most commonly used treatment for infection with *F. psychrophilum*. Oxytetracycline (OTC) has been used worldwide (Post 1987; Branson 1998; Groff and LaPatra 2001;

LaFrentz and Cain 2004; Lumsden et al. 2006), and amoxicillin and oxolinic acid have been used throughout Europe (Branson 1998; Bruun et al. 2000). Several studies suggest that antimicrobial resistance is occurring in treated populations (Schrag and Wiener 1995; Brunn et al. 2000). Starting in 1986, oxolinic acid was used to treat *F. psychrophilum* in Denmark hatcheries, but by 2000 the bacteria was 100% resistant to oxolinic acid. Between 1994 and 1998, 60 to 75% of *F. psychrophilum* in Danish hatcheries showed resistance to both OTC and amoxicillin (Brunn et al. 2000). Another potential treatment option for BCWD is vaccination, and though development of a vaccine has been attempted (Sudheesh and Cain 2016), none are currently commercially available.

Due to concerns about antibiotic resistance and the lack of a vaccine, other strategies to prevent *F. psychrophilum* infections warrant investigation. Hadidi et al. (2008) suggested using a genetically resistant brood fish to manage BCWD outbreaks. In 2005, the US Department of Agriculture-Agricultural Research Service's (USDA-ARS) National Center for Cool and Cold Water Aquaculture (NCCWA) developed a program to create a Rainbow Trout strain that was genetically resistant to *F. psychrophilum* (Hadidi et al. 2008; Leeds et al. 2010). A similar approach has been used to manage Rainbow Trout in the presence of the whirling disease parasite, *Myxobolus cerebralis*. Researchers at Colorado Parks and Wildlife (CPW) developed *M. cerebralis*-resistant strains of Rainbow Trout by crossing a domesticated, *M. cerebralis*-resistant strain with wild strains of Rainbow Trout (Schisler et al. 2006; Fetherman et al. 2012). These crosses result in Rainbow Trout that are resistant to *M. cerebralis* and retain important wild characteristics (Fetherman et al. 2011), including the ability to survive and breed in the wild (Fetherman et al. 2014).

Using *F. psychrophilum*-resistant fish in Colorado's state hatchery system may provide a valuable tool to reduce mortality due to outbreaks of BCWD in the hatchery, and these *psychrophilum*-resistant Rainbow Trout, or PRR, were brought into the state in 2016 for that purpose. However, it is unknown whether and to what degree the PRR are also resistant to *M. cerebralis*. *M. cerebralis* was introduced to Colorado in the late 1980s, and later found in free-ranging salmonid populations in 11 of the state's 15 major river drainages (Barney et al. 1988; Nehring and Thompson 2003), resulting in the population

collapse of wild Rainbow Trout fisheries throughout Colorado (Nehring and Thompson 2001). The state of Colorado has been using *M. cerebralis*-resistant Rainbow Trout to reestablish populations in the presence of the parasite (Fetherman et al. 2014; Avila et al. 2018), and reproduction and recruitment are occurring (Fetherman et al. 2014). Stocking *F. psychrophilum*-resistant fish with no resistance to *M. cerebralis* into streams and rivers in Colorado could result in failure due to mortality associated with *M. cerebralis* exposure, as well as increased infection severity and loss of progress gained from *M. cerebralis*-resistant Rainbow Trout stocking efforts. For *F. psychrophilum*-resistant fish to be a viable management tool in Colorado, it is imperative to determine if the PRR exhibit any resistance to *M. cerebralis*, understand if resistance to both *F. psychrophilum* and *M. cerebralis* is compatible and achievable, and, given the possibility for exposure to either or both pathogens in a hatchery or wild environment, understand how they might interact when dual exposure occurs.

The overall goal of the two experiments described here was to determine if crossing strains of Rainbow Trout resistant for each pathogen would result in a trout strain that is genetically resistant to both pathogens. The goal of the first experiment was to determine if it was possible to develop dual resistance by crossing a Rainbow Trout resistant to *M. cerebralis* (multigenerational cross) with the PRR. Not only was the resistance to each pathogen individually of interest, but I also wanted to understand the possible effects of coinfection when fish were exposed to both pathogens in a specific order because fish that are infected with *F. psychrophilum* can be stocked into the wild to prevent high losses within the hatchery and then can be exposed to *M. cerebralis*; or an *M. cerebralis* positive hatchery may have fish exposed to it first followed shortly after by *F. psychrophilum* exposure. Experiments with *M. cerebralis* have shown that first-generation versus multi-generation fish maintain a higher resistance (Fetherman et al. 2012). Therefore, the goal of my second experiment was to determine if it is possible to develop a first-generation Rainbow Trout cross that is resistant to *F. psychrophilum* when crossing pure parental strains resistant to either *F. psychrophilum* or *M. cerebralis*.

METHODS

Rainbow Trout strains and crosses

Three strains of *M. cerebralis*-resistant Rainbow Trout were used across both experiments, German Rainbow Trout, Harrison Lake Rainbow Trout, and the German Rainbow x Harrison Lake Rainbow Trout (Table 2.1). The pure German Rainbow (GR) is a domesticated hatchery Rainbow Trout which was exposed to *M. cerebralis* over many generations in Germany and is more resistant to *M. cerebralis* than many other Rainbow Trout strains found in North America (Hedrick et al. 2003). Due to its domestication, it was believed that the survival of the German Rainbow Trout in the wild would be lower than other Rainbow Trout that were historically stocked into Colorado (Schisler et al. 2006). Experimental crosses between the GR and other Rainbow Trout strains have led to Rainbow Trout that are *M. cerebralis*-resistant and survive in the wild (Fetherman et al. 2014; Avila et al. 2018). The Harrison Lake Rainbow Trout (HL; origin: Harrison Lake, Montana; Wagner et al. 2006), is one of the wild Rainbow Trout strains that were used to cross with the GR (Schisler and Fetherman 2009; Fetherman et al. 2015). The HL has shown resistance to *M. cerebralis* which was developed due to rapid natural selection (Miller and Vincent 2008). The cross between the GR and HL (GRxHL) resulted in *M. cerebralis* resistance and has shown survival in the wild (Fetherman et al. 2015). The GR x HL used within Experiment 1 are 87.5% GR and 12.5% HL and has been propagated as a hatchery brood stock since 2006 (Schisler et al. 2011). Despite showing *M. cerebralis* resistance, the GR x HL shows some of the highest mortality in the Colorado Parks and Wildlife hatchery system due to *F. psychrophilum* infections (Eric Fetherman; personal communication).

In 2005, the US Department of Agriculture-Agricultural Research Service's National Center for Cool and Cold Water Aquaculture developed a selective breeding program to create a Rainbow Trout strain that was genetically resistant to *F. psychrophilum* (Hadidi et al. 2008; Leeds et al. 2010). The strains used to create the *F. psychrophilum*-resistant population were chosen based on known genetic history and history domestication including the Ennis NFH Shasta strain; College of southern Idaho, House Creek strain; Kamloops/Puget Sound Steelhead cross; and University of Washington, Donaldson

strain (Silverstein et al. 2009). These *F. psychrophilum*-resistant Rainbow Trout strains are known as the ARS-Fp-R strain (Table 2.1; Leeds et al. 2010; Wiens et al. 2013). A third-generation lot of ARS-Fp-R was sent to Utah Division of Wildlife Resources, and then in 2016, Colorado Parks and Wildlife imported the *F. psychrophilum*-resistant Rainbow Trout from Utah Division of Wildlife Resources to be used in the Colorado Parks and Wildlife hatchery system to manage mortality due to *F. psychrophilum* infections. Within the Colorado Parks and Wildlife hatchery system, these imported fish are known as *psychrophilum*-resistant rainbow (PRR; Table 2.1). As of 2020, the most current ARS-Fp-R line of Rainbow Trout being produced by the USDA-ARS NCCWA is the fifth generation of *F. psychrophilum*-resistant selected Rainbow Trout (Greg Wiens; personal communication). In these experiments I used the PRR from Colorado and the fifth generation ARS-Fp-R from the National Center for Cool and Cold Water Aquaculture to investigate *F. psychrophilum* resistance.

Experiment 1 – Dual Exposure to Flavobacterium psychrophilum and Myxobolus cerebralis

Two strains and one cross of Rainbow Trout were used for this dual exposure experiment (Table 2.1; Figure 2.1), the PRR, which is resistant to *F. psychrophilum*, the GR x HL, which is resistant to *M. cerebralis,* and the cross of the GR x HL and PRR (GHP), which was evaluated for maintaining resistance to both pathogens. These strains were spawned at the Colorado Parks and Wildlife Crystal River Hatchery (Carbondale, Colorado) in January 2019 and then transported as eyed eggs to the Colorado Parks and Wildlife Bellvue Fish Research Hatchery (Bellvue, Colorado) for hatching. Fish were moved from the Colorado Parks and Wildlife Bellvue Fish Research Hatchery, eight weeks post-hatch, to a lab located on Colorado State University (CSU) main campus. Fish were moved two days prior to the beginning of the experiment. Fish were counted into transportation bags (35 fish per bag) by strain and cross to ensure the same number of fish per tank. Transportation bags were half filled with water (~2.5 gallons) and topped off with pure oxygen prior to being closed. Bags were transported from the Colorado Parks and Wildlife Bellvue Fish Research Hatchery in coolers to the CSU Anatomy/Zoology building and then carried into the lab. Bags were placed in assigned tanks for each strain/cross and treatment (see below). Bags were acclimated by temperature and water mixing prior to pouring fish into the tank.

There were six exposure treatments for each Rainbow Trout variety: (1) no pathogen exposure (control), (2) mock injection, (3) *F. psychrophilum* only exposure, (4) *M. cerebralis* only exposure, (5) *F. psychrophilum* exposure followed by *M. cerebralis* exposure four days later, and (6) *M. cerebralis* exposure followed by *F. psychrophilum* exposure four days later. The 18 experimental treatments (6 exposures x 3 strains) had 6 replicates each, resulting in 108 total twenty-gallon tanks. Each fish tank held thirty-five individual Rainbow Trout of the assigned strain/cross, resulting in 3,780 fish in the experiment. Water (13.4 ºC ± 2.1 SD) was sourced from the city and dechlorinated by running through large, activated charcoal filters. Fish tanks were set up for flow-through water exchange and each tank was set for a flow of 15 gallons of water per hour.

To limit potential cross-contamination from pathogen-exposed tanks, control tanks were located on the top shelf of the three-tier shelving system. Strains were randomly assigned to tanks and either a control or mock injection treatment within the top shelf of the system. Pathogen exposure treatment and strain were then randomly assigned to the remaining tanks on the top shelf (note that control/mock injection tanks were never located next to pathogen exposure tanks on the same shelving unit) and the tank on the other two tiers of the shelving system.

Flavobacterium psychrophilum culture and preparation is described in Appendix 2.1. Prior to moving fish from the Colorado Parks and Wildlife Bellvue Fish Research Hatchery, an initial batch weight of each tank was taken and used to calculate the total amount of feed per day (g) and the dose of *F. psychrophilum* for each tank (PRR: 0.46 g ± 0.03 SD; GR x HL: 0.41g ± 0.05; GHP: 0.48 g ± 0.06). For *F. psychrophilum* exposure, Rainbow Trout were first sedated using MS-222 (90 mg per mL of water) and then injected subcutaneously at the dorsal midline posterior to the dorsal fin with 8.8×10^6 colony forming units per milliliter (CFU/mL) of virulent *F. psychrophilum* (CSF259-93 obtained from Dr. Kenneth Cain, Moscow ID, 25 µL; Appendix 2.1). For the mock injection, Rainbow Trout were similarly subcutaneously injected with 25 µL of tryptone yeast extracts and salt (TYES) to ensure that exposure to the bacteria and not physical injury from injection caused mortality (Appendix 2.1). No

injections occurred for fish in the control, *M. cerebralis* only, or, initially, the *M. cerebralis* exposure followed by *F. psychrophilum* treatments.

Myxobolus cerebralis triactinomyxons (TAMs), the waterborne infectious stage of the parasite, were produced by *Tubifex tubifex* worm cultures maintained at the Colorado Parks and Wildlife Parvin Lake Research Station (Red Feather Lakes, Colorado). The concentration of viable TAMs was estimated by mixing 1000 µL of filtrate containing TAMs and 60 µL of crystal violet; 84.6 µL of this mixture was then placed on a slide and the number of TAMs per slide was counted. Ten TAM counts were conducted out of the filtrate to get an average number of TAMs per mL, and fish (732.2 ± 34.6 degree-days [ºC*days] post-hatch) were exposed to 2,000 TAMs per individual for a total of 70,000 TAMs per tank (following Fetherman et al. 2011). Flow to each tank was stopped prior to the addition of TAMs, and each tank received aeration to ensure mixing of the TAMs. The amount of filtrate needed to deliver 2,000 TAMs per fish was placed in each tank in two doses, one after another, each dose containing half of the necessary filtrate. Using two doses helped ensure equal distribution of TAMs in the tank and accounted for a possible unequal distribution of TAMs within the filtrate. Flow was resumed after 1 hour of exposure. Control tanks were treated the same way as exposure tanks. Fish in the control treatment, mock injection, *F. psychrophilum* only, and, initially, *F. psychrophilum* exposure followed by *M. cerebralis* exposure were not exposed to TAMs.

On the first day of the experiment, mock injections were conducted first to prevent accidental exposures to *F. psychrophilum* using the same injectors, followed by *F. psychrophilum* treatments (*F. psychrophilum* only, *F. psychrophilum* and then *M. cerebralis*) and *M. cerebralis* treatments (*M. cerebralis* only, *M. cerebralis* and then *F. psychrophilum*). The same exposure methods described above were used for the dual exposures, but some were exposed to *F. psychrophilum* and then *M. cerebralis* four days later (18 tanks) or exposed to *M. cerebralis* and then *F. psychrophilum* four days later (18 tanks).

Experiment 1 had two experimental objectives. The first was to conduct an *F. psychrophilum* exposure experiment to observe mortality. *Flavobacterium psychrophilum* exposures were conducted on day zero and day four and fish were held for 28-days so that mortality can stabilize (showing no more

increased mortality) because fish can survive and recover from *F. psychrophilum* infections. The second part started after the completion of the *F. psychrophilum* portion of the experiment. All remaining fish were reared until they reached 2,000-degree days (2,347.8 ± 73.3 SD) which was necessary to ensure full development of myxospores in the treatments with *M. cerebralis*.

Tanks were cleaned every two weeks on a rotating schedule. Throughout the rearing process all tanks were monitored twice daily and moribund and dead fish in each tank were measured, weighed, signs of disease were documented, and the fish were then removed. Fish were fed twice a day at the standardized feeding rate (percent body weight per day [% BW/d]) based on the manufacturer's (BioOregon) suggested specifications of fish size and rearing temperature. Feed amount was adjusted daily based on the number of fish within each tank and water temperature. Batch weights were taken from fish in each tank by placing all fish from the tank into a tared water bucket on a scale, obtaining individual weights by dividing the total weight by the known number of fish, and calculating the grams per fish. The batch weight was used to assign a feeding rate (% BW/d) and calculate total amount of feed per day (g) based on fish number for each tank. Batch weights and adjustment of feeding rate was conducted every two weeks starting 28-days post exposure to not affect mortality results in the classic *F. psychrophilum* exposure experiment by handling fish.

After 2,000-degree days, all surviving fish were euthanized, weighed, measured, and inspected for clinical signs of whirling disease and/or bacterial coldwater disease. All euthanized fish had their heads removed from the body just behind the operculum and pectoral fins and placed in individually labeled bags and frozen (Fetherman et al. 2012). Myxospores were enumerated (O'Grodnick 1975) using pepsin–trypsin digest (PTD; Markiw and Wolf, 1974a,b). The processing of fish was initiated at the Colorado Parks and Wildlife Aquatic Animal Health Laboratory (AAHL; Brush, Colorado) and conducted by the Aquatic Animal Health Laboratory staff. A subset of samples were processed entirely by the Aquatic Animal Health Laboratory, including pepsin-trypsin digestion and myxospore counting. The remaining fish were digested using pepsin at the Aquatic Animal Health Laboratory and then transferred to the Colorado Cooperative Fish and Wildlife Research Unit laboratory to finish trypsin

digestion and myxospore counting. The same methods were used in both labs to ensure consistency in the results.

Statistical Analysis

The statistical analysis focused on five endpoints: (1) 28-day post exposure mortality, (2) end of experiment mortality, (3) differences in growth, (4) disease sign, and (5) myxospore counts for M. cerebralis exposed tanks.

Mortality

Cumulative percent mortality (CPM), the number of dead fish divided by total number of fish at the start, was calculated for each tank for the first 28 days post-exposure and at the end of the experiment. A Chi-squared test was used to determine if there were differences in mortality (a proportion) between the strains (Rainbow Trout variety), treatments, and strain by treatment interaction (strain*treatment). If there was evidence of a difference in mortality, pairwise comparisons with a Tukey adjustment were used to compare between strain and treatments.

Growth

The difference in weight at 28-days post-exposure was analyzed using ANOVA with strain, treatment, and strain*treatment as the factors to explain differences in growth due to exposure to *F. psychrophilum*. The difference in weight at the end of the experiment was analyzed using an ANCOVA with strain, treatment, and interaction between strain and treatment as the factors, and number of fish within a tank at the end of the experiment as a covariate as tank density was thought to explain differences in growth. Difference in weight at the end of the experiment may also show if *F. psychrophilum* affects growth after surviving *F. psychrophilum* infections since traditional *F. psychrophilum* exposure experiments are only conducted for 28-days. If there was evidence of a difference in growth in either model then a pairwise comparison with a Tukey adjustment was implemented. Type III sums of squares were used to account for the unbalanced number of tanks and statistical significance was inferred at the alpha = 0.05 level.

Myxospore counts

To account for myxospore counts of zero in the control, mock injection, and *F. psychrophilum* only exposure, a two-part modeling approach was taken. First, I used a logistic regression to quantify the difference in myxospore counts among the tanks not exposed to *M. cerebralis* to those that were exposed to *M. cerebralis*. The response is specified by a binary variable (0 if not exposed to *M. cerebralis* or 1 if a fish was exposed to *M. cerebralis*) with the predictor variables of strain, treatment, and the strain by treatment interaction. Chi-squared values were then used to determine if there were statistical difference in number of myxospores between the predictor variables. Second, I used a negative-binomial regression, which helped account for over dispersion of the data (Duan et al. 1983; Boulton and Williford 2018), to compare the average difference in myxospore count among strains, treatments, and their interaction in only fish that were exposed to *M. cerebralis*. If there was evidence of differences in myxospore counts, then a pairwise comparison with a Tukey adjustment was used to compare among strains and treatments.

Clinical signs of whirling disease

Clinical disease signs included cranial deformities, spinal deformities, opercular deformities, exophthalmia, lower jaw deformities, and blacktail. Due to gas bubble disease being present at the end of the experiment, exophthalmia was removed from the analysis; no additional effects of mortality were observed due to gas bubble disease. A logistic regression was used to fit the data with proportion of individuals in a tank as the response and strain, treatment, and the interaction between strain and treatment as the factors that predict clinical signs of disease. If there was evidence of a difference in clinical signs of disease, then a pairwise comparisons with a Tukey adjustment was used to compare among strains and treatments.

Experiment 2

F1-generation crosses were created by crossing pure German Rainbow (GR) and pure Harrison Lake Rainbow Trout (HL), which are resistant to *M. cerebralis*, with ARS-Fp-R obtained from the US Department of Agriculture-Agricultural Research Service's National Center for Cool and Cold Water Aquaculture or PRR obtained from Colorado Parks and Wildlife. All strains were created in collaboration

with the US Department of Agriculture-Agricultural Research Service's National Center for Cool and Cold Water Aquaculture, the Colorado Parks and Wildlife Crystal River Hatchery, and the Colorado Parks and Wildlife Bellvue Fish Research Hatchery. The National Center for Cool and Cold Water Aquaculture provided ARS-Fp-R milt and the Crystal River Hatchery provided PRR milt, and milt from these sources were crossed with pure German Rainbow and pure Harrison Lake Rainbow Trout eggs at the Colorado Parks and Wildlife Bellvue Fish Research Hatchery in January 2020. All F1 crosses were made using *F. psychrophilum*-resistant males and *M. cerebralis*-resistant females. The spawning resulted in first-generation HL x PRR, HL x ARS-Fp-R, GR x PRR, and GR x ARS-Fp-R crosses (Table 2.1; Figure 2.1). The Crystal River Hatchery produced pure PRR Rainbow Trout, whereas the National Center for Cool and Cold Water Aquaculture produced the ARS-Fp-R and an *F. psychrophilum*-susceptible line Rainbow Trout (S-Line), and both facilities shipped eyed eggs to the Bellvue Fish Research Hatchery where they were hatched.

Fish were moved from the Colorado Parks and Wildlife Fish Research Hatchery to a lab located on Colorado State University (CSU) main campus in the basement of the Anatomy/Zoology building, using the same transportation method described for Experiment 1. An initial sample weight of each tank was taken prior to moving fish (HL: 0.78 g ± 0.06 (SD); HL x PRR: 1 g ± 0; HL x ARS-Fp-R: 1 g ± 0; GR: 1 g ± 0; GR x PRR: 1.18 g ± 0.06; GR x ARS-Fp-R: 1.24 g ± 0.09; PRR: 1.46 g ± 0.10; S-Line: 1.1 g ± 0.14; ARS-Fp-R: 1.1 g ± 0.12). Weights were used to calculate the total amount of feed per day (g) for each tank and to calculate *F. psychrophilum* dosage. Control fish were randomly assigned to tanks located on the top shelf of the three-tier shelving system to limit potential bacterial contamination of control tanks, and *F. psychrophilum* treatment tanks were randomly assigned to the remaining tanks on the lower two tiers. Each twenty-gallon tank held twenty-five individual Rainbow Trout of the assigned strain/cross. Water (10 ºC ± 0.77 SD) was sourced from the city and dechlorinated by running through large, activated charcoal filters. Fish tanks were set up for flow-through water exchange and each tank was set for a flow of 15 gallons of water per hour.

I compared nine Rainbow Trout varieties, five pure strains, GR, HL, ARS-Fp-R, PRR, and the S-Line, and four F1-generation crosses, GR x ARS-Fp-R, HL x ARS-Fp-R, GR x PRR, and HL x PRR. All nine Rainbow Trout varieties in the exposure treatment were injected subcutaneously posterior to the dorsal fin above the midline, with virulent 8.8×10^6 CFU/mL of *F. psychrophilum* (CSF259-93, 25 μL), and each strain had ten replicates (tanks) except for the GR x ARS-Fp-R (five tanks) which had high mortalities in the hatchery, and the S-Line Rainbow Trout (two tanks). Mock injections were given to four strains (GR, HL, ARS-Fp-R, and HL x ARS-Fp-R), with two replicates each, and injected similarly with tryptone yeast extract and salts (TYES, 25 μL). I did not include mock injections for every strain or equal numbers of replicates because water resources were limited in the laboratory. Fish were monitored twice a day after injections. Moribund and dead fish in each tank were removed and recorded for 28-days after injection. At the end the rearing period, all remaining fish were euthanized.

Statistical Analysis

Cumulative percent mortality (CPM) of all replicates were calculated for each strain and treatment throughout the 28-day experimental period. A Chi-squared test was used to determine if there was a relationship between cumulative mortality and strain, exposure, and the interaction between strain and exposure (strain*exposure) and if there was a relationship between mortality and strain dependent on fish weight (strain*weight). If there was evidence of a difference in mortality, then Tukey adjusted pairwise comparisons were used to compare among strains and treatments. Finally, a logistic regression was used to estimate the probability of mortality based on factors that significantly affected cumulative percent mortality identified in the Chi-square analysis.

RESULTS

Experiment 1

Development of F. psychrophilum exposure

After subcutaneous injections, I saw bruising around the injection side in both mock injection and *F. psychrophilum* exposure treatments. Bruising would either disappear in one to two days or progress into a red inflammation at the injection site. After inflammation, it was common to observe a boil around

the injection site but boils also formed without associated bruising and inflammation. Once inflammation or boils formed, most fish stopped eating and displayed lethargic swimming behavior. After feeding slowed, anal fecal stalactites were observed on many fish in the *F. psychrophilum* exposure treatments. Boils at the injection site would typically increase in size until they ruptured, resulting in the classical bacterial coldwater disease lesions with yellow bacteria around the edges. Bacteria were also seen surrounding the anal and pelvic fins and anus.

Mortality

The earliest mortalities within a treatment occurred between five- and ten-days post-exposure to *F. psychrophilum* and concluded 7-14 days after they began (Figure 2.2). Cumulative mortality curves for the fish given mock injections (TYES) differed greatly from those exposed to *F. psychrophilum* indicating that mortality in the *F. psychrophilum* exposures occurred due to the bacteria and not from the injections themselves. Mortality in the *M. cerebralis* followed by *F. psychrophilum* exposure treatment started four to five days after other treatments due to the time lag in exposure to *F. psychrophilum*. Although the CPM curves were relatively similar within a strain/cross for fish in the *F. psychrophilum* only and *F. psychrophilum* followed by *M. cerebralis* exposure treatments, the GHP experienced fairly rapid mortality in the *M. cerebralis* followed by *F. psychrophilum* exposure treatment, suggesting that exposure to the two pathogens in that order had higher mortality rates for this strain. The control treatment had no mortality and the *M. cerebralis*-only exposure had only two mortalities at 28-days post-exposure.

Mean CPM at 28-days post exposure ranged between 32% and 98.6% for *F. psychrophilum* exposure treatments (*F. psychrophilum* only, *F. psychrophilum* followed by *M. cerebralis* exposure, and *M. cerebralis* followed by *F. psychrophilum* exposure; Table 2.2). The Chi-squared test indicated an interaction between strain and treatment ($\chi^2 = 70.17$, p-value < 0.01). For all three strains, mortality was low and did not differ among the control, TYES, and *M. cerebralis* only treatments (Table 2.2). Mortality at 28 days was significantly higher in *F. psychrophilum* exposure treatments compared to controls, TYES, and *M. cerebralis* treatments. Mortality did not differ between the GR x HL strain and GHP cross when

exposed to *F. psychrophilum.* However, mortality for the PRR was lower than the other two varieties when exposed to *F. psychrophilum* (Table 2.2).

The mean mortality at the end of the experiment ranged between 9% and 100% (Table 2.3). The Chi-squared test indicated an interaction between strain and treatment ($\chi^2 = 428.12$, p-value < 0.01). Although the GHP cross and GR x HL strain did not differ in overall mortality at the end of the experiment, the PRR strain showed lower mortality (Table 2.3; Figure 2.3). Mortality was higher in *F. psychrophilum* treatments compared to control, TYES, and *M. cerebralis* treatments for the GHP cross, GR x HL strain and PRR strain. Dual exposures resulted in higher mortality compared to *F. psychrophilum* only exposure and *M. cerebralis* only for the PRR strain but not the other two strains (Table 2.3, Figure 2.3).

Growth

The mean weights 28-days post-exposure ranged from 0.65 to 1.35 grams. The ANOVA indicated evidence of a strain by treatment interaction (F = 2.57, p-value < 0.01). Pairwise comparisons indicated that the GHP control was 0.7 grams heavier at 28 days post exposure than the other strains and treatments (Table 2.4).

Mean weights at the end of the experiment ranged between 27.89 and 140.81 grams (Figure 2.4). Density did not affect the final weight among treatments or strains (p-value = 0.16). However, there was a strain and treatment interaction (F = 5.15, p-value < 0.01). The larger fish on average spanned the *F. psychrophilum* only and *F. psychrophilum* followed by *M. cerebralis* exposure treatments from the GHP and PRR strains (Figure 2.4). The largest fish belonged to the GHP cross in the *M. cerebralis* followed by *F. psychrophilum* exposure treatment.

Myxospore counts

As expected, all control, *F. psychrophilum* only, and TYES treatments for every strain had no myxospores in any individual (Table 2.5). Mean myxospores per Rainbow Trout variety in the *M. cerebralis* exposure treatments (*M. cerebralis* only, *F. psychrophilum* followed by *M. cerebralis* exposure, and *M. cerebralis* followed by *F. psychrophilum* exposure) ranged between 555 and 645,201

per fish. The Chi-squared test indicated that there were differences between treatments exposed to *M. cerebralis* and those that were not exposed (χ^2 = 106.45, p-value < 0.01). The probability of having a myxospore count greater than zero was 99.9% when exposed to *M. cerebralis* compared to a probability of zero when not exposed to *M. cerebralis* (logistic regression). The negative-binomial regression indicated a strain*treatment interaction (χ^2 = 22.80, p-value < 0.01). There were statistically significant differences among the PRR, GHP, and GR x HL when exposed to *M. cerebralis* only. The GR x HL strain developed the lowest number of myxospores, followed by GHP and then PRR (Table 2.5, Figure 2.5). When fish survived exposure to *F. psychrophilum*, myxospore counts on average were lower in the *F. psychrophilum* followed by *M. cerebralis* exposure treatment compared to the *M. cerebralis* only treatment (Figure 2.5). For the GHP, exposure to *M. cerebralis* followed by exposure to *F. psychrophilum* resulted in significantly higher myxospore counts.

Clinical signs of whirling disease

Clinical signs of whirling disease (cranial deformities, spinal deformities, opercular deformities, lower jaw deformities, and blacktail) developed between two- and three-months post-exposure. Both the GHP cross and PRR strain exhibited classic whirling swimming behavior when fish were startled. The PRR strain had the most visible signs of whirling disease, with a significantly higher proportion of blacktail visible compared to the GR x HL and GHP (Figure 2.6) and showed extreme spinal deformities developing between two- and three-months post-exposure. Four out of the five clinical signs of disease (cranial deformities, spinal deformities, opercular deformities, lower jaw deformities) showed evidence of a strain by treatment interaction (p-value < 0.01). Within each Rainbow Trout variety, no fish in *F. psychrophilum* only treatments developed a higher percentage of deformities than control fish.

Experiment 2

Mortalities started within the first two days post-exposure for the ARS-Fp-R and S-Line strains compared to the other strains, which started between three- and five-days post-exposure (Figure 2.7). There were no mortalities within the mock injection controls.

Chi-squared tests indicated that fish weight did not have an effect on mortality among strains (strain*weight; $\chi2 = 9.68$, p-value = 0.08) and no interaction between strain and exposure ($\chi2 = 0$, p-value = 1). However, there was a difference in mortality by both strain and exposure ($\chi2 = 544.95$ (strain); $\chi2 = 493.10$ (exposure), p-value < 0.01 for strain and exposure). The TYES control groups showed no mortality associated with injection. The estimated probabilities of mortality for the strains in the F. psychrophilum exposure ranged between 19.3 and 98.8% (Table 2.6). The S-Line, which was used as a positive control, showed expected high mortality (Figure 2.8). The S-Line mortality was not different from that of the pure Harrison Lake or pure German Rainbow strains (Figure 2.8), indicating that the M. cerebralis-resistant strains are not resistant to F. psychrophilum, which is similar to experiment one. The PRR strain had significantly less mortality compared to the ARS-Fp-R strain (Figure 2.8), indicating that the PRR is more resistant to F. psychrophilum than ARS-Fp-R. The GR x ARS-Fp-R and GR x PRR showed less mortality than the pure German strain and similar to that of the ARS-Fp-R (Figure 2.8) suggesting that some resistance was transferred from the F. psychrophilum resistant strains and indicating F. psychrophilum resistance in the F1 generation. The HL x ARS-Fp-R and HL x PRR showed less mortality than the pure Harrison Lake strain (Figure 2.8) suggesting resistance was transferred from the F. psychrophilum-resistant strains. In addition, the HL x ARS-Fp-R and HL x PRR showed the lowest mortality compared to the pure German Rainbow, pure Harrison Lake, the GR x ARS-Fp-R, and the GR x PRR, suggesting that F. psychrophilum resistance can be passed on to F1 progeny. The HL x PRR and the PRR strains had the lowest mortality compared to the other nine, indicating the highest F. psychrophilum resistance among the strains.

DISCUSSION

The overall objective of these experiments was to evaluate the potential of developing a Rainbow Trout that was resistant to both *M. cerebralis* and *F. psychrophilum*, suitable for use in Colorado's state hatchery system and for stocking in aquatic systems in which *M. cerebralis* is established. I investigated the consequences of infection with each pathogen on three Rainbow Trout varieties, coinfection with both pathogens in these varieties, and *F. psychrophilum* exposure effects in pure strains and F1 generation

crosses. It appears that some crosses might be useful in the development of Rainbow Trout that are resistant to both pathogens. However, others do not appear to have that potential. Strains known for their resistance to *M. cerebralis* were not resistant to *F. psychrophilum*, and vice versa, strains known for their resistance to *F. psychrophilum* were not resistant to *M. cerebralis*. The intermediate cross did not appear to be resistant to either pathogen. Despite the resistance characteristics of any given trout strain, coinfection led to an increase in average mortality for all strains compared to single-pathogen exposure.

It appears that some Rainbow Trout crosses have greater promise for creating dual resistance than others. The results of the second experiment indicate that *F. psychrophilum*-resistance can be maintained in first-generation crosses, with the HL x PRR exhibiting the lowest mortality from *F. psychrophilum* exposure. These crosses may provide another management tool for fisheries managers, similar to the benefits of using *M. cerebralis*-resistant Rainbow Trout. Use of the HL x PRR may reduce or eliminate the need for antibiotics, as the probability of mortality from *F. psychrophilum* exposure was less than 20%. Additionally, since the Harrison Lake Rainbow Trout originates from a wild Rainbow Trout population (Wagner et al. 2006), the HL x PRR may also show better survival and reproduction after being stocked compared to the PRR, since PRR are domesticated (Silverstein et al. 2009) and may not do well in the wild. I did not assess how the HL x PRR performed in the presence of *M. cerebralis* in the second experiment because evaluation of *M. cerebralis* resistance was not possible due to time constraints involved in the development of the parasite. However, the HL x PRR may be a good candidate for the development of dual resistance to *F. psychrophilum* and *M. cerebralis*. Prior research has shown that the pure Harrison Lake Rainbow Trout produce fewer myxospores than I observed in the GHP, GR x HL, and PRR (Schisler et al. 2011); additional research into *M. cerebralis* resistance in Har x PRR would be required to determine if the Har x PRR retains *M. cerebralis* resistance.

The HR x PRR or HR x ARS-Fp-R may still be a viable option in the development of dual resistance to both *M. cerebralis* and *F. psychrophilum*. The reduction in mortality associated with *F. psychrophilum* exposure was not as large as that seen in the HL x PRR but was still significant. Additionally, first generation Rainbow Trout cross offspring of the pure German Rainbow and the

Colorado River Rainbow Trout (CRR) have shown high resistance to *M. cerebralis* (Fetherman et al. 2012) suggesting that both GR x PRR or GR x ARS-Fp-R may retain resistance to *M. cerebralis*. However, resistance to *M. cerebralis* in the GR x PRR and GR x ARS-Fp-R should still be confirmed.

Although it appears that dual resistance may be possible with some strains, the lack of response in other strains indicates that dual resistance may be difficult to develop. The GHP showed no resistance to either pathogen as single exposures to *F. psychrophilum* and dual exposures to *F. psychrophilum* and *M. cerebralis* resulted in high mortalities (> 75%) and high myxospore counts. High mortality and high myxospore counts indicate that the GHP is not a good candidate for developing dual resistance, particularly because it seems to have lost resistance to both pathogens. Currently it is unknown which genes provide resistance to *F. psychrophilum* (personal communication, Greg Wiens, National Center for Cool and Cold Water Aquaculture). Development of the *F. psychrophilum*-resistant Rainbow Trout used selective breeding at the US Department of Agriculture-Agricultural Research Service's National Center for Cool and Cold Water Aquaculture (Hadidi et al. 2008; Silverstein et al. 2009; Leeds et al. 2010; Wiens et al. 2013). However, genetic parentage analyses were not done, and the mechanism of genetic resistance may depend on the specific parent strains and genes that allow for disease resistance. The German Rainbow Trout strain is highly resistant to *M. cerebralis* with 9 ± 5 genes estimated to confer genetic resistance (Fetherman et al. 2012). A possible reason for the GHP to show little to no resistance to *F. psychrophilum* is that the German Rainbow genes that confer resistance to *M. cerebralis* may negatively interact with the genes that infer *F. psychrophilum* resistance (Lhorente et al. 2014; Fraslin et al. 2020).

I included the ARS-Fp-R Rainbow Trout strain to determine if it had higher *F. psychrophilum* resistance than the PRR, because it had undergone more generations of selection (three for the PRR versus five for the ARS-Fp-R) and was predicted to show similar or lower mortality due to the generational differences in *F. psychrophilum*-resistance selection (Greg Wiens National Center for Cool and Cold Water Aquaculture, personal communications). The lower mortality of the PRR strain indicates that it has higher resistance to *F. psychrophilum* and the additional selection in the ARS-Fp-R Rainbow

Trout strain did not confer greater resistance. One explanation is that there were other environmental variables not accounted for. For example, transportation of eyed eggs from West Virginia to Colorado may have induced additional stress due to temperature and pressure changes, resulting in increased mortality. Both the ARS-Fp-R and the S-Line, which were spawned in West Virginia and then sent to Colorado, showed mortality beginning around day two of the experiment which is slightly earlier than the traditional time frame seen in all other Rainbow Trout strains. Another explanation for the differences in mortality between the strains is the continuous exposure to *F. psychrophilum* in the Colorado Parks and Wildlife hatchery system that may have allowed the PRR to develop increased resistance compared to the ARS-Fp-R Rainbow Trout. Similar continuous exposure is believed to have produced the *M. cerebralis* genetic resistance in the German Rainbow Trout (Hedrick et al. 2003). Based on these results, there is no need to replace the current PRR brood stock with another *F. psychrophilum*-resistant brood stock with more generations of selection in the Colorado Parks and Wildlife hatchery system.

A concerning and unexpected observation was the relatively high average myxospore counts for the *M. cerebralis*-resistant GR x HL. The high number of myxospores found in the GR x HL indicate a loss of resistance in this strain and could be attributed to backcrossing or lack of exposure to the parasite. Outcrossing and/or backcrossing may have occurred in the hatchery and resulted in decreased genetic resistance to *M. cerebralis*. The observed myxospore counts were roughly the same as those seen in second-generation backcrosses of F1-generation crosses of the German Rainbow and CRR (Fetherman et al. 2012). A loss of resistance could also be the result of the absence of *M. cerebralis* in the hatchery system. In a single hatchery generation, the expression of hundreds of genes in Rainbow Trout can be altered, resulting in selection of traits that are beneficial in the hatchery but not in the wild (Christie et al. 2012, 2016). The absence of the parasite could therefore reduce selection for resistance to *M. cerebralis* given that those genes are not needed for survival in the hatchery environment. The loss of resistance to *M. cerebralis* in the GR x HL strain is concerning for future management and reintroduction efforts. Stocking fish that are susceptible to the parasite could result in less successful survival and recruitment of

Rainbow Trout (Avila et al 2018). Additionally, these fish could produce high numbers of myxospores, which may lead to increased *M. cerebralis* in wild systems.

The PRR strain showed no resistance to *M. cerebralis* and had 3.45 times more myxospores than the highly susceptible CRR (Fetherman et al. 2011). The GHP had similar numbers of myxospores as the CRR, also indicating no genetic resistance to *M. cerebralis*. High numbers of myxospores and high mortality indicated that neither the PRR nor GHP strains are good candidates for stocking into *M. cerebralis*-positive waters. Stocking these strains could result in increased *M. cerebralis* and loss of fish due to *M. cerebralis* infection.

Co-infection with *F. psychrophilum* and *M. cerebralis* increased CPM for every Rainbow Trout strain. Similar trends have been seen with parasite and bacterial co-infections of Rainbow Trout in previous experiments with increased mortality compared to single-pathogen exposure (Busch et al. 2003; Bandilla et al. 2006). Ma et al. (2019) also showed higher mortality in Rainbow Trout with co-infections of *F. psychrophilum* and infectious hematopoietic necrosis virus (IHNV), compared to those infected with a single pathogen. Currently, it is not known what factor(s) increase mortality due to co-infection or the specific interactions between *M. cerebralis* and *F. psychrophilum*. It is thought that reduced apparent post-stocking survival may be due to domestication of the fish being stocked, movement of the fish out of the study area, environmental conditions, and/or predation (Schisler et al. 2006; Avila et al. 2018). Another possible reason for reduced post-stocking survival may be co-infection of fish in the hatchery or in the aquatic environment post-stocking. Co-infections are common within the hatchery and wild environments due to exposure to heterogeneous infectious pathogens (Kotob et al. 2016; Ma et al. 2019). Reducing disease exposure in hatcheries by changing or improving husbandry protocols may not only reduce disease outbreaks but increase long-term survival within the hatchery and/or post-stocking survival.

My results indicate that the GHP was not genetically resistant to either pathogen and would not be a good candidate for use in hatchery production or wild stocking. This may be because the GR x HL currently used in the hatchery system is not entirely resistant to *M. cerebralis* and may not be a good

candidate for developing a Rainbow Trout with dual resistance. However, it is possible to create an F1-generation cross that is genetically resistant to *F. psychrophilum*, which gives hope for the development of dual resistance. Increased resistance to *F. psychrophilum* in the HL x PRR and HL x ARS-Fp-R suggests that dual resistance may be possible if the correct parent strains are used for crossing. However, we do not know the resistance of the HL x PRR or HL x ARS-Fp-R to *M. cerebralis*. Future whirling disease experiments will need to be conducted to evaluate if these crosses can be used to manage *M. cerebralis*. If these crosses do indeed have dual resistance, we are one step closer to managing potential coinfections of detrimental pathogens in the aquaculture setting as well as in the wild.

Table 2.1. The different Rainbow Trout strains (German Rainbow x Harrison Lake, GR x HL; *psychrophilum*-resistant rainbow, PRR; Harrison Lake , HL; German Rainbow, GR; S-Line, ARS-Fp-S; Agricultural Research Service - *F. psychrophilum*-resistant, ARS-Fp-R) and first generation crosses (GR x HL x *psychrophilum*-resistant rainbow, GHP; Harrison Lake x *psychrophilum*-resistant rainbow, HL x PRR; Harrison Lake x Agricultural Research Service - *F. psychrophilum* - Resistant, HL x ARS-Fp-R; GR x *psychrophilum*-resistant rainbow, GR x PRR; German Rainbow x Agricultural Research Service - *F. psychrophilum* - Resistant, GR x ARS-Fp-R) used for each experiment and known pathogen resistance.

Rainbow Trout Strains/Crosses	Abbreviation	Experiment	Resistance	Fish Type
German Rainbow x Harrison Lake	GR x HL	1	*M. cerebralis*	Strain
(German Rainbow x Harrison Lake) x *psychrophilum*-resistant rainbow	GHP	1	Unknown	F1-generation cross
psychrophilum-resistant rainbow	PRR	1, 2	*F. psychrophilum*	Strain
Harrison Lake	HL	2	*M. cerebralis*	Strain
Germain Rainbow	GR	2	*M. cerebralis*	Strain
S-Line	ARS-Fp-S	2	Unknown	Strain
Agricultural Research Service - *F. psychrophilum* - Resistant	ARS-Fp-R	2	*F. psychrophilum*	Strain
Harrison Lake x *psychrophilum*-resistant rainbow	HL x PRR	2	Unknown	F1-generation cross
Harrison Lake x Agricultural Research Service - *F. psychrophilum* - Resistant	HL x ARS-Fp-R	2	Unknown	F1-generation cross
German Rainbow x *psychrophilum*-resistant rainbow	GR x PRR	2	Unknown	F1-generation cross
German Rainbow x Agricultural Research Service - *F. psychrophilum* - Resistant	GRx ARS-Fp-R	2	Unknown	F1-generation cross

Table 2.2. Mean cumulative percent mortality (CPM) and 95% confidence intervals (CIs) for each strain and treatment (TYES = mock injection, Fp = *Flavobacterium psychrophilum* only, Mc = *Myxobolus cerebralis* only, FpMc = exposed to *F. psychrophilum* followed by *M. cerebralis*, and McFp = exposed to *M. cerebralis* followed by *F. psychrophilum*) in Experiment 1 at 28-days post-exposure.

Strain	Treatment	Mean CPM	95% CI
GR x HL	Control	0	[0, 0]
GR x HL	TYES	0.10	[0.064, 0.15]
GR x HL	Fp	0.90	[0.80, 1]
GR x HL	Mc	0.0095	[0, 0.025]
GR x HL	FpMc	0.94	[0.92, 0.96]
GR x HL	McFp	0.99	[0.96, 1]
GHP	Control	0	[0, 0]
GHP	TYES	0.014	[0, 0.031]
GHP	Fp	0.68	[0.38, 0.97]
GHP	Mc	0.0048	[0, 0.017]
GHP	FpMc	0.82	[0.66, 0.98]
GHP	McFp	0.88	[0.60, 1]
PRR	Control	0	[0, 0]
PRR	TYES	0.095	[0, 0.21]
PRR	Fp	0.32	[0.068, 0.58]
PRR	Mc	0	[0, 0]
PRR	FpMc	0.46	[0.40, 0.52]
PRR	McFp	0.58	[0.27, 0.90]

Table 2.3. Mean cumulative percent mortality and 95% confidence intervals (CIs) for each Rainbow Trout variety (Strain) and treatment at the end of Experiment 1.

Strain	Treatment	Mean CPM	95% CI
GR x HL	Control	0.057	[0.038, 0.076]
GR x HL	TYES	0.17	[0.13, 0.22]
GR x HL	Fp	0.96	[0.91, 1]
GR x HL	Mc	0.12	[0.051, 0.20]
GR x HL	Fp Mc	0.99	[0.97, 1]
GR x HL	Mc Fp	1	[1, 1]
GHP	Control	0.091	[0.032, 0.15]
GHP	TYES	0.10	[0.064, 0.15]
GHP	Fp	0.77	[0.44, 1]
GHP	Mc	0.20	[0.011, 0.38]
GHP	Fp Mc	0.96	[0.92, 1]
GHP	Mc Fp	0.99	[0.98, 1]
PRR	Control	0.1	[0.044, 0.16]
PRR	TYES	0.20	[0.0873 0.30]
PRR	Fp	0.45	[0.17, 0.73]
PRR	Mc	0.63	[0.38, 0.87]
PRR	Fp Mc	0.84	[0.74, 0.94]
PRR	Mc Fp	0.79	[0.57, 1]

Table 2.4. Mean weight (g) for each Rainbow Trout variety (Strain) and treatment at 28-day post-expousre with coresponding 95% confidence intervals for Experiment 1.

Strain	Treatment	Mean Weight	95% CI
GR x HL	Control	1.030	[0.96, 1.098]
GR x HL	TYES	0.79	[0.69, 0.89]
GR x HL	Fp	0.97	[0.67, 1.27]
GR x HL	Mc	0.75	[0.66, 0.85]
GR x HL	Fp Mc	0.76	[0.39, 1.13]
GR x HL	Mc Fp	0.79	[0, 4.91]
GHP	Control	1.35	[1.25, 1.44]
GHP	TYES	0.88	[0.74, 1.024]
GHP	Fp	0.96	[0.79, 1.14]
GHP	Mc	0.89	[0.78, 0.99]
GHP	Fp Mc	0.99	[0.82, 1.16]
GHP	Mc Fp	0.65	[0.27, 1.020]
PRR	Control	0.94	[0.90, 0.98]
PRR	TYES	0.85	[0.81, 0.90]
PRR	Fp	0.90	[0.81, 0.99]
PRR	Mc	0.83	[0.76, 0.90]
PRR	Fp Mc	0.87	[0.75, 0.98]
PRR	Mc Fp	0.84	[0.73, 0.94]

Table 2.5. Mean myxospore count per head and 95% confidence intervals (CIs) for each Rainbow Trout variety (Strain) and treatment (TYES = mock injection, Fp = *Flavobacterium psychrophilum* only, Mc = *Myxobolus cerebralis* only, FpMc = exposed to *F. psychrophilum* followed by *M. cerebralis*, and McFp = exposed to *M. cerebralis* followed by *F. psychrophilum*) included in Experiment 1. NA denotes no data available because of 100% mortality before end of experiment.

Strain	Treatment	Mean Spores	95% CI
GR x HL	Control	0	[0, 0]
GR x HL	TYES	0	[0, 0]
GR x HL	Fp	0	[0, 0]
GR x HL	Mc	77569	[54351, 100788]
GR x HL	FpMc	556	[0, 2947]
GR x HL	McFp	NA	NA
GHP	Control	0	[0, 0]
GHP	TYES	0	[0, 0]
GHP	Fp	0	[0, 0]
GHP	Mc	224553	[168762, 280344]
GHP	FpMc	89964	[0, 210861]
GHP	McFp	497539	[497539, 497539]
PRR	Control	0	[0, 0]
PRR	TYES	0	[0, 0]
PRR	Fp	0	[0, 0]
PRR	Mc	645201	[309353, 981049]
PRR	FpMc	388942	[179097, 598787]
PRR	McFp	563697	[382138, 745257]

Table 2.6. The estimated probability of mortality for the strains and crosses exposed to *F. psychrophilum* (Fp) or given a mock injection (TYES) with corresponding 95% confidence intervals (CIs) from Experiment 2.

Strain	Treatment	Probability of Mortality	95% CI.
ARS-Fp-R	Fp	0.60	[0.54, 0.66]
ARS-Fp-R	TYES	0	[0, 0]
HL	Fp	0.88	[0.83, 0.91]
HL	TYES	0	[0, 0]
HL x ARS-Fp-R	Fp	0.48	[0.42, 0.54]
HL x ARS-Fp-R	TYES	0	[0, 0]
HL x PRR	Fp	0.19	[0.15, 0.25]
HL x PRR	TYES	0	[0, 0]
GR	Fp	0.999	[0.96, 1]
GR	TYES	0	[0, 0]
GR x ARS-Fp-R	Fp	0.72	[0.63, 0.79]
GR x ARS-Fp-R	TYES	0	[0, 0]
GR x PRR	Fp	0.76	[0.70, 0.81]
GR x PRR	TYES	0	[0, 0]
PRR	Fp	0.23	[0.18, 0.28]
PRR	TYES	0	[0, 0]
S-Line	Fp	0.98	[0.87, 1]
S-Line	TYES	0	[0, 0]

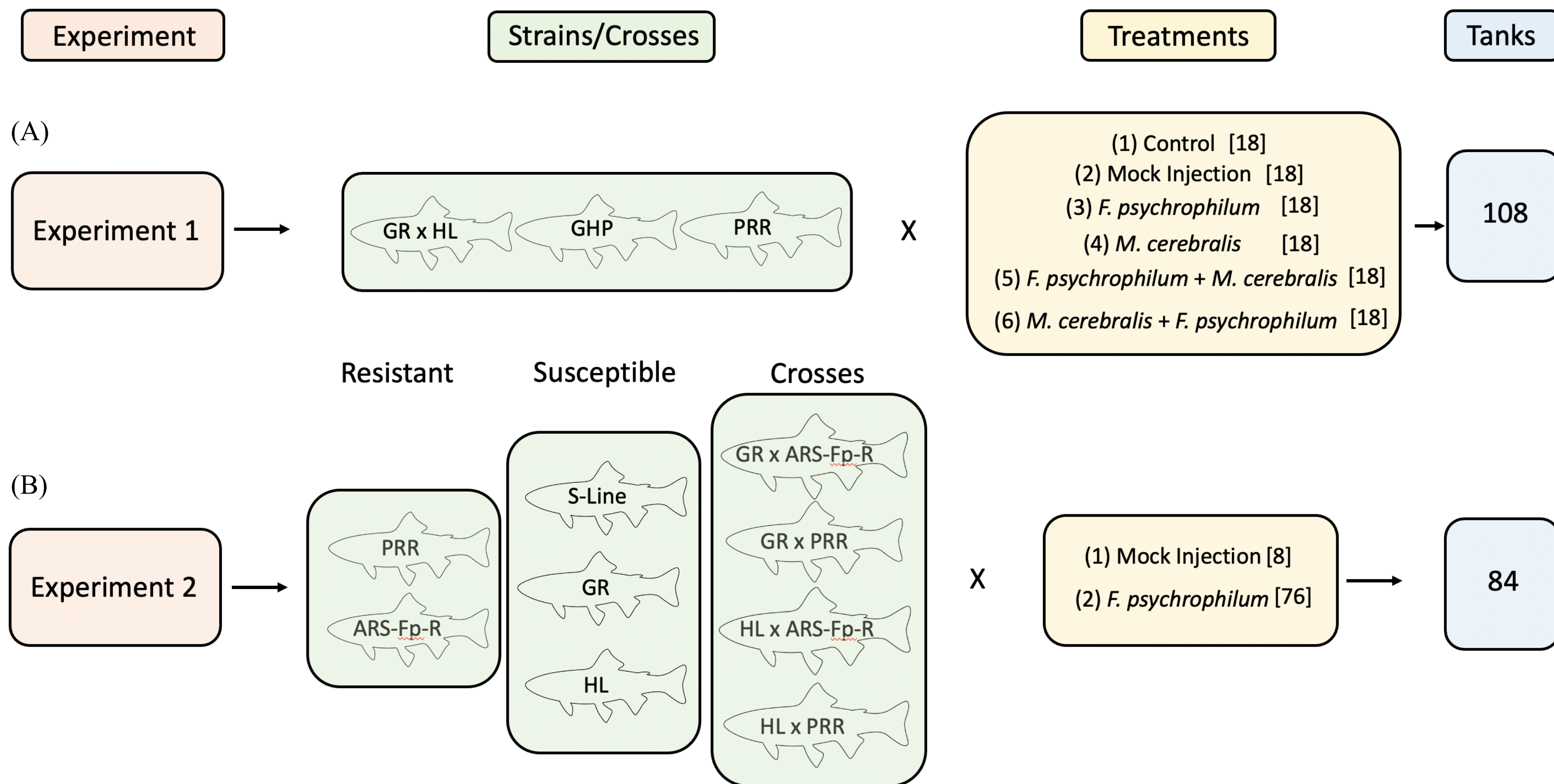

Figure 2.1. Experimental design for Experiment 1 (A) and Experiment 2 (B). Three total strains or crosses were used in Experiment 1, the GR x HL, PRR, and GHP. There were six treatments (control, mock injection, *F. psychrophilum* only, *M. cerebralis* only, *F. psychrophilum* first and the *M. cerebralis* four days later, and *M. cerebralis* first and then *F. psychrophilum* four days later) used in Experiment 1. The number of tanks for each treatment are denoted by [ ]. A total of 108 tanks were used in Experiment 1. Nine total strains or cross were used in Experiment 2, the PRR, ARS-Fp-R, S-Line, GR, HL, GR x ARS-Fp-R, GR x PRR, HL x ARS-Fp-R, and HL x PRR. Only two treatments (mock injection and *F. psychrophilum* only) were used Experiment 2. A total of 84 tanks were used in Experiment 2.

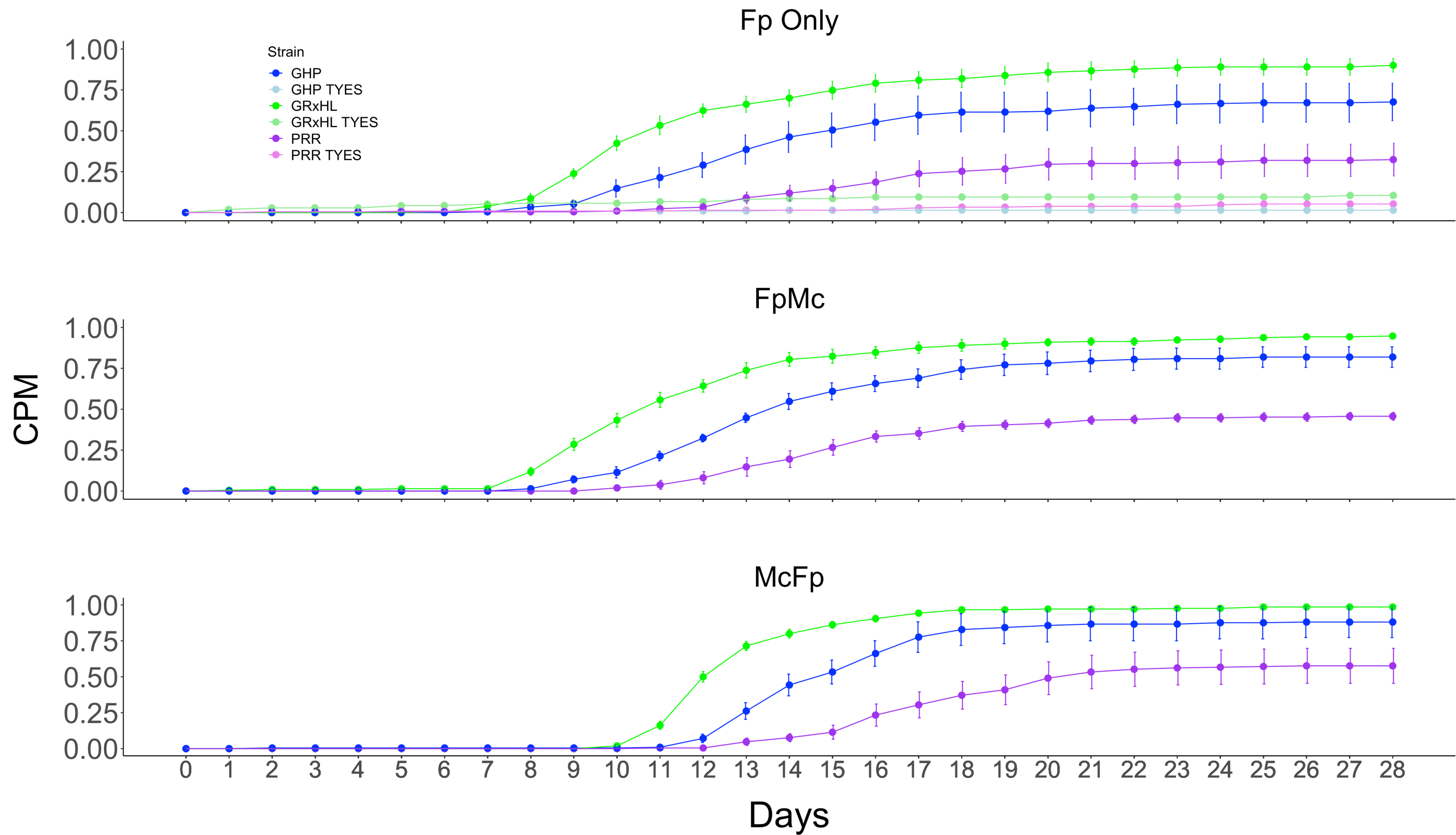

Figure 2.2. Cumulative percent mortality (CPM; standard error bars) in the first 28 days of Experiment 1 for each strain/cross exposed to *Flavobacterium psychrophilum* only (Fp Only), *F. psychrophilum* followed by *Myxobolus cerebralis* (FpMc), or *M. cerebralis* followed by *F. psychrophilum* (McFp). Mortalities by strain in the mock injection treatment (TYES) are shown in relation to the Fp Only treatment.

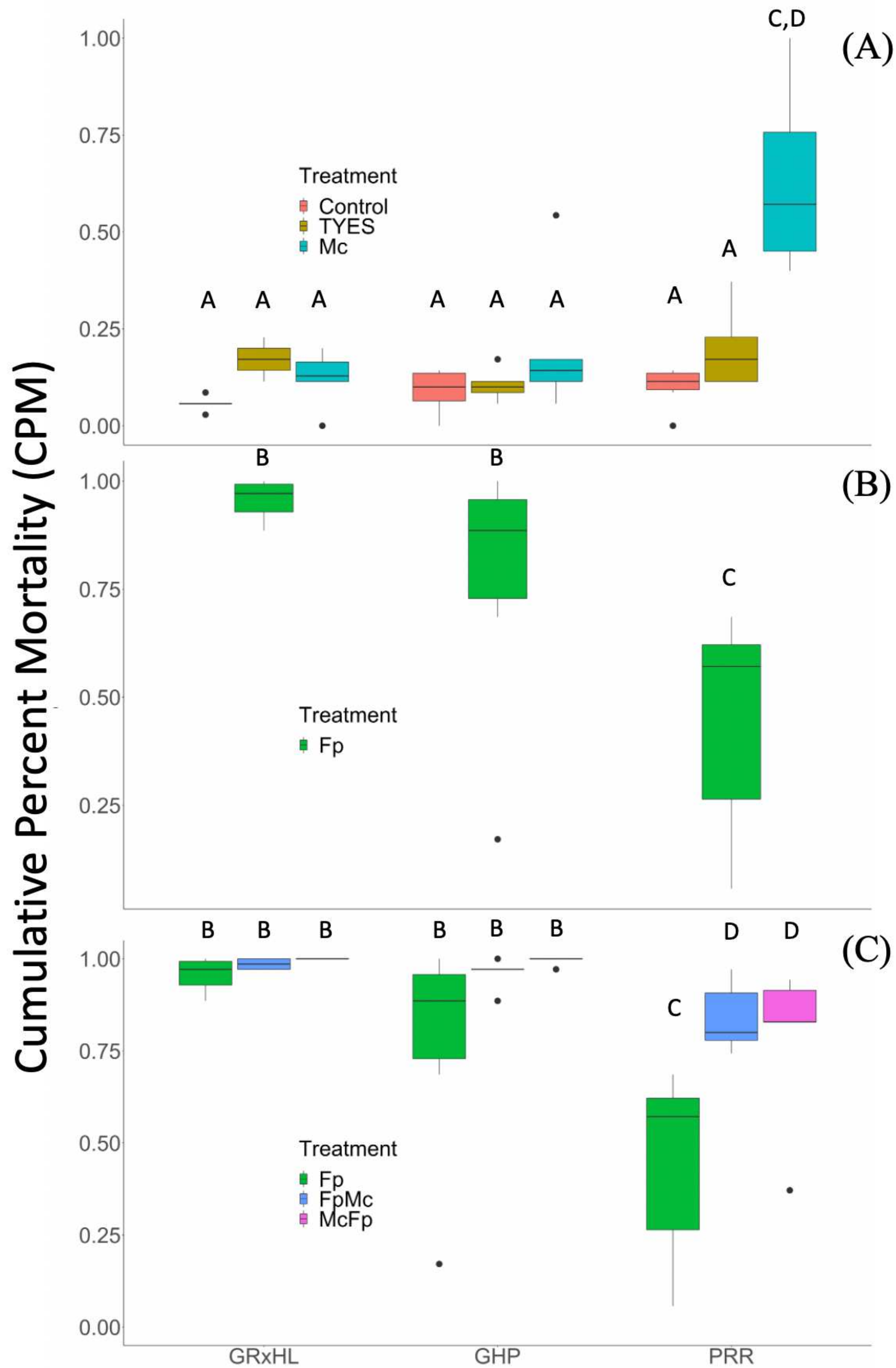

Figure 2.3. Cumulative percent mortality (CPM) by strain and treatment (A) Control, TYES = mock injection, and Mc = *Myxobolus cerebralis* only; (B) Fp = *Flavobacterium psychrophilum* only; (C) Fp = *Flavobacterium psychrophilum* only, FpMc = exposed to *F. psychrophilum* followed by *M. cerebralis*, and McFp = exposed to *M. cerebralis* followed by *F. psychrophilum*, at the end of Experiment 1. Black lines within the boxes indicate the median of the distribution. Box and whisker plots with the same letter indicate no significant differences and box and whisker plots with different letters indicate statistically significant differences.

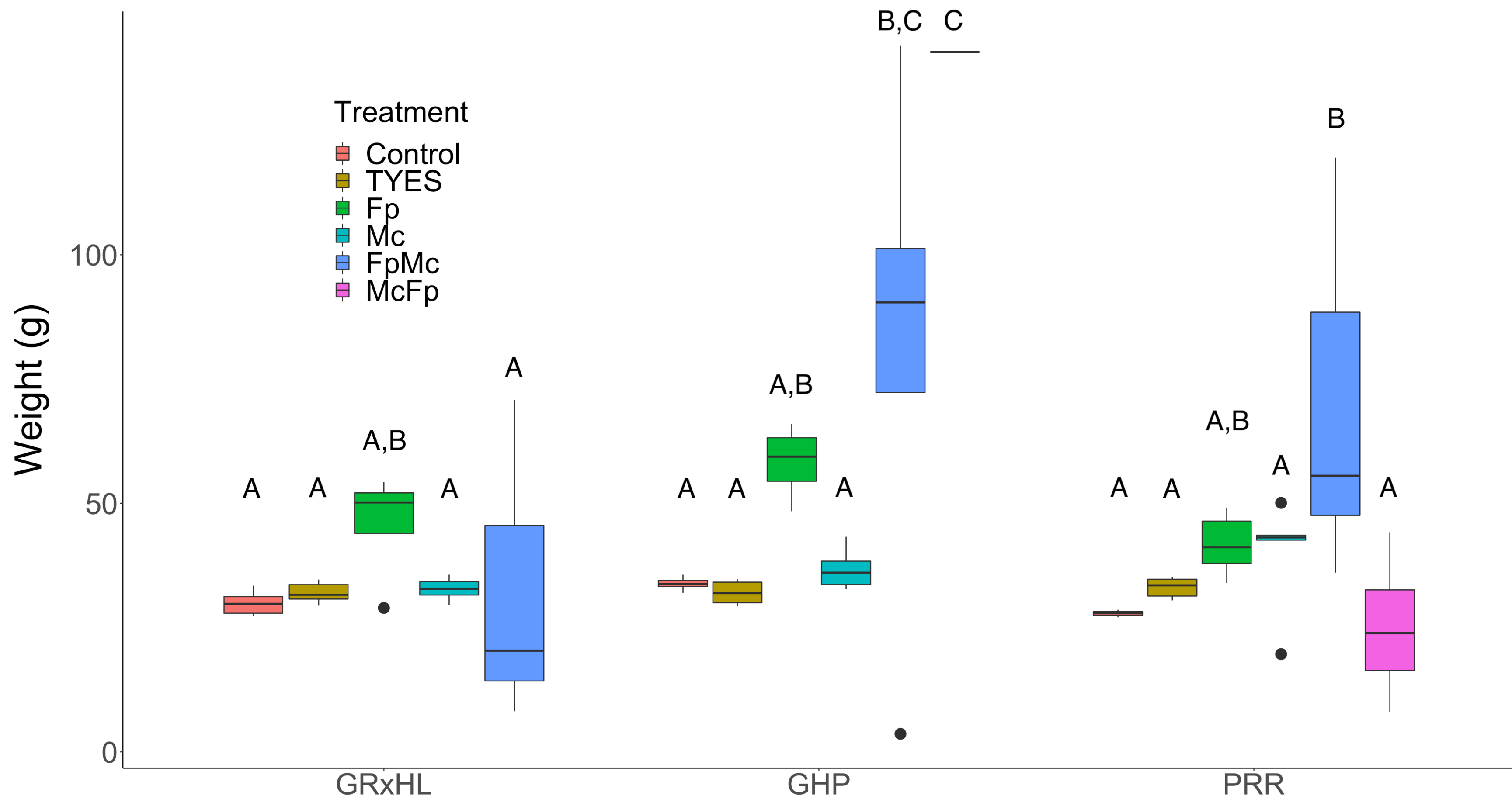

Figure 2.4. Weight (g) by strain and treatment (TYES = mock injection, Fp = *Flavobacterium psychrophilum* only, Mc = *Myxobolus cerebralis* only, FpMc = exposed to *F. psychrophilum* followed by *M. cerebralis*, and McFp = exposed to *M. cerebralis* followed by *F. psychrophilum*) at the end of Experiment 1. NA denotes no data available because 100% mortality occurred before the end of the experiment for HxH McFp treatment. Black lines within the boxes indicate the median of the distribution. Box and whisker plots with the same letter indicate no significant differences and box and whisker plots with different letters indicate statistically significant differences.

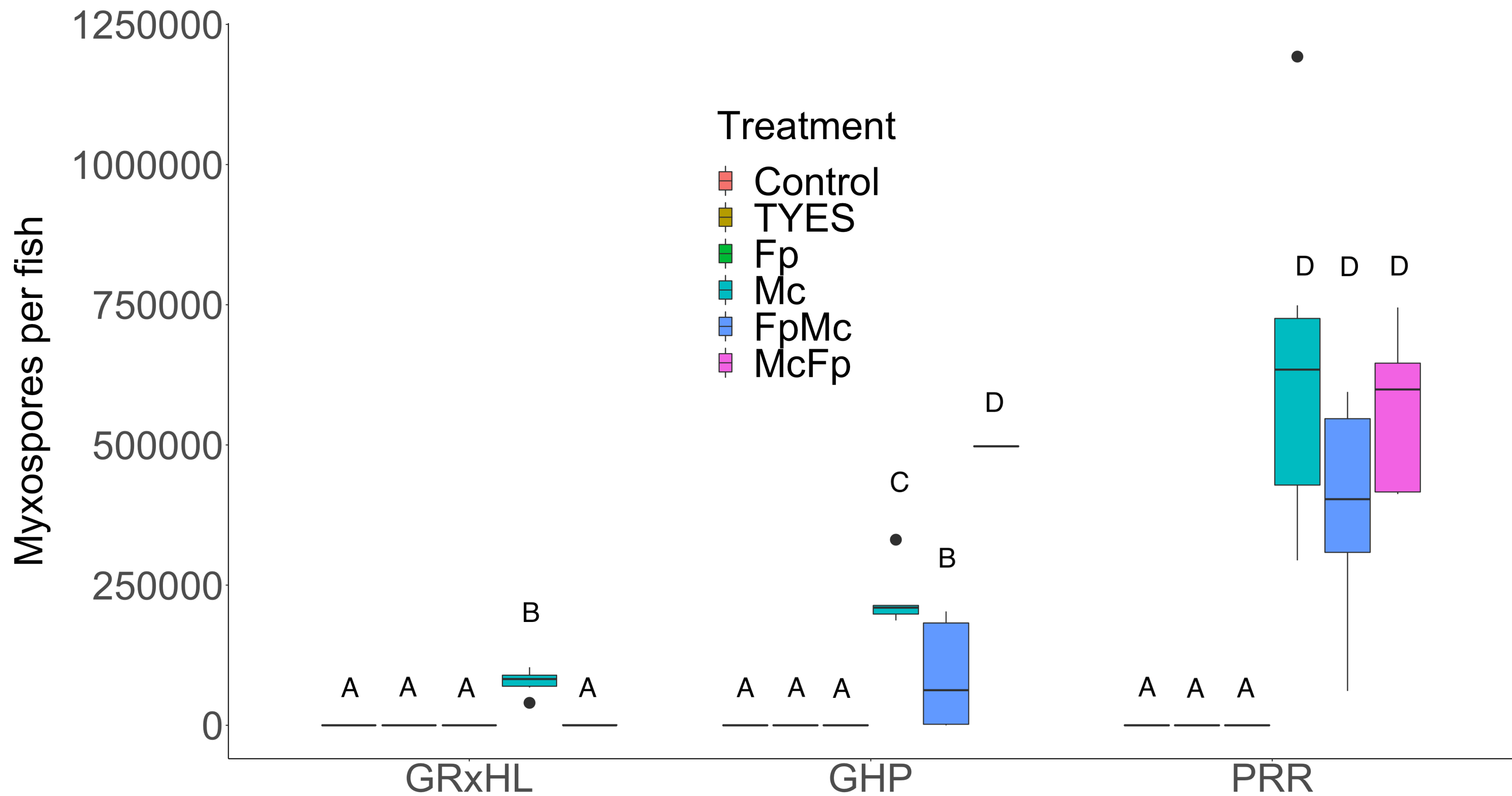

Figure 2.5. Mean myxospore count per head by Rainbow Trout variety and individual treatment (TYES = mock injection, Fp = *Flavobacterium psychrophilum* only, Mc = *Myxobolus cerebralis* only, FpMc = exposed to *F. psychrophilum* followed by *M. cerebralis*, and McFp = exposed to *M. cerebralis* followed by *F. psychrophilum*) at the end of Experiment 1. NA denotes no data available because of 100% mortality before end of experiment for HxH McFp treatment. Black lines within the boxes indicate the median of the distribution. Box and whisker plots with the same letter indicate no significant differences and box and whisker plots with different letters indicate statistically significant differences.

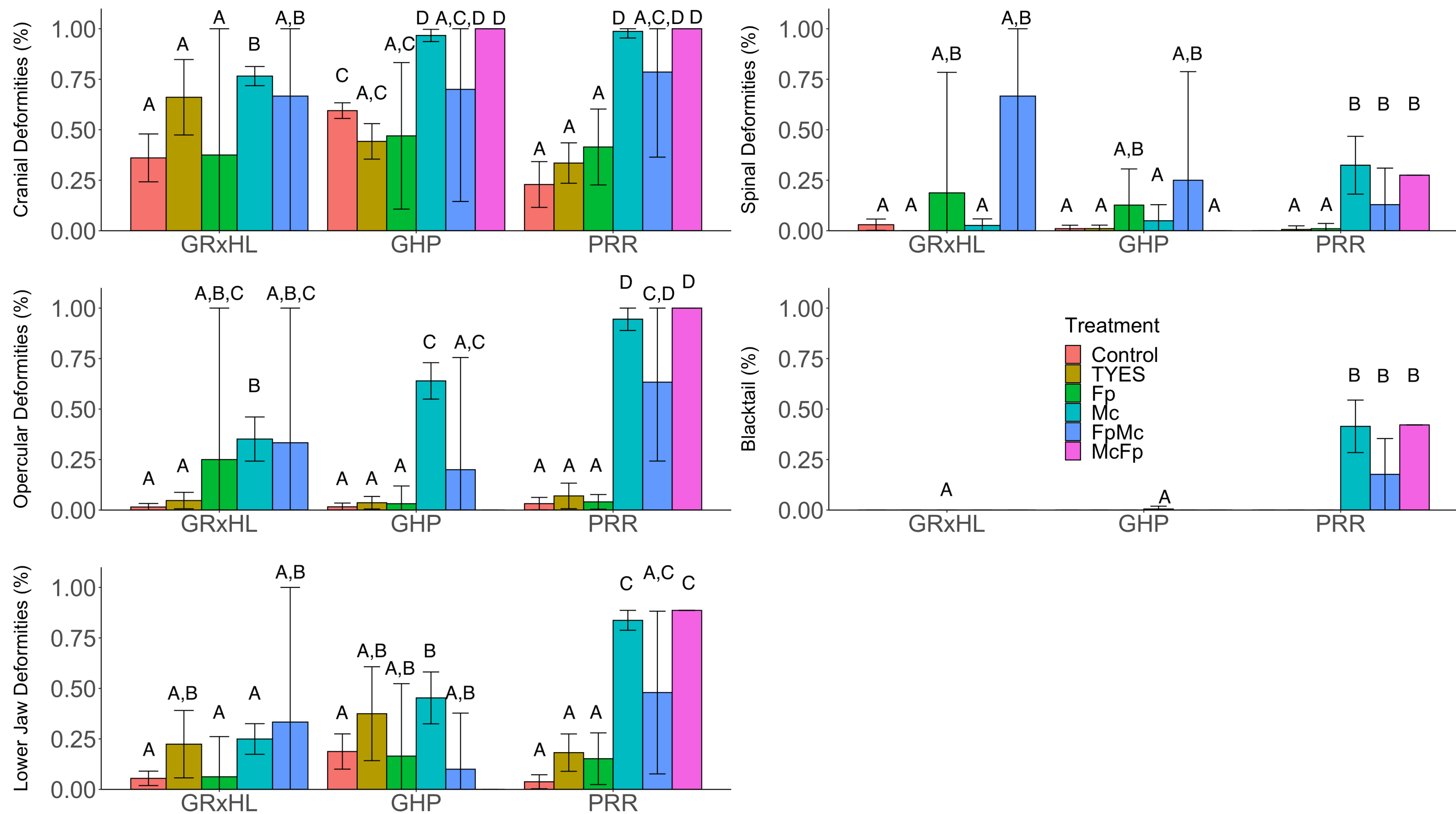

Figure 2.6. Percent of deformed individuals (standard error bars) exhibiting cranial deformities, spinal deformities, opercular deformities, lower jaw deformities, and blacktail by strain and treatment (TYES = mock injection, Fp = *Flavobacterium psychrophilum* only, Mc = *Myxobolus cerebralis* only, FpMc = exposed to *F. psychrophilum* followed by *M. cerebralis*, and McFp = exposed to *M. cerebralis* followed by *F. psychrophilum*) at the end of Experiment 1. NA denotes no data available for the HxH McFp treatment because of 100% mortality before end of experiment.

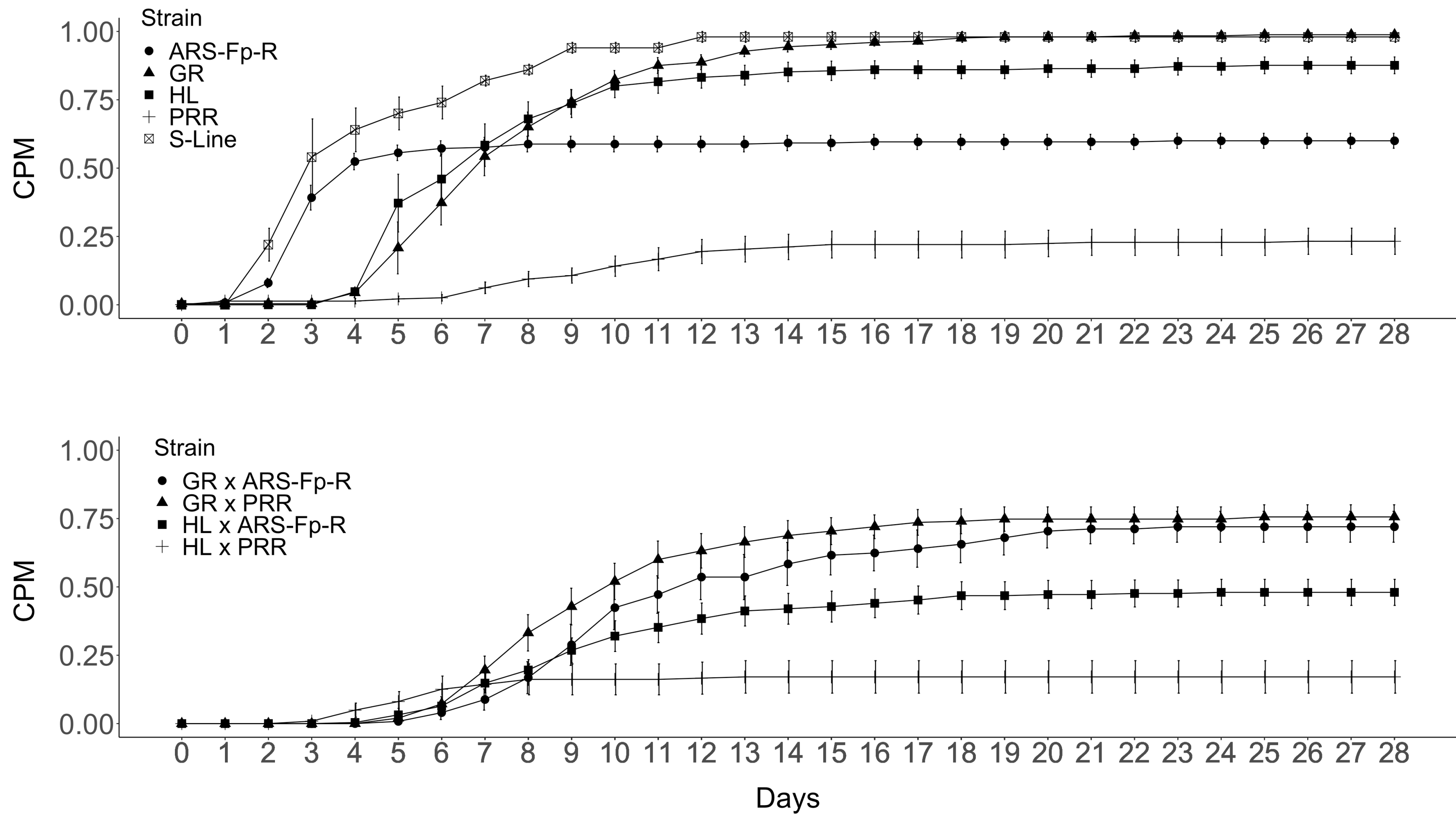

Figure 2.7. Cumulative percent mortality (CPM; standard error bars) in the first 28 days of Experiment 2 for each strain exposed to *Flavobacterium psychrophilum*.

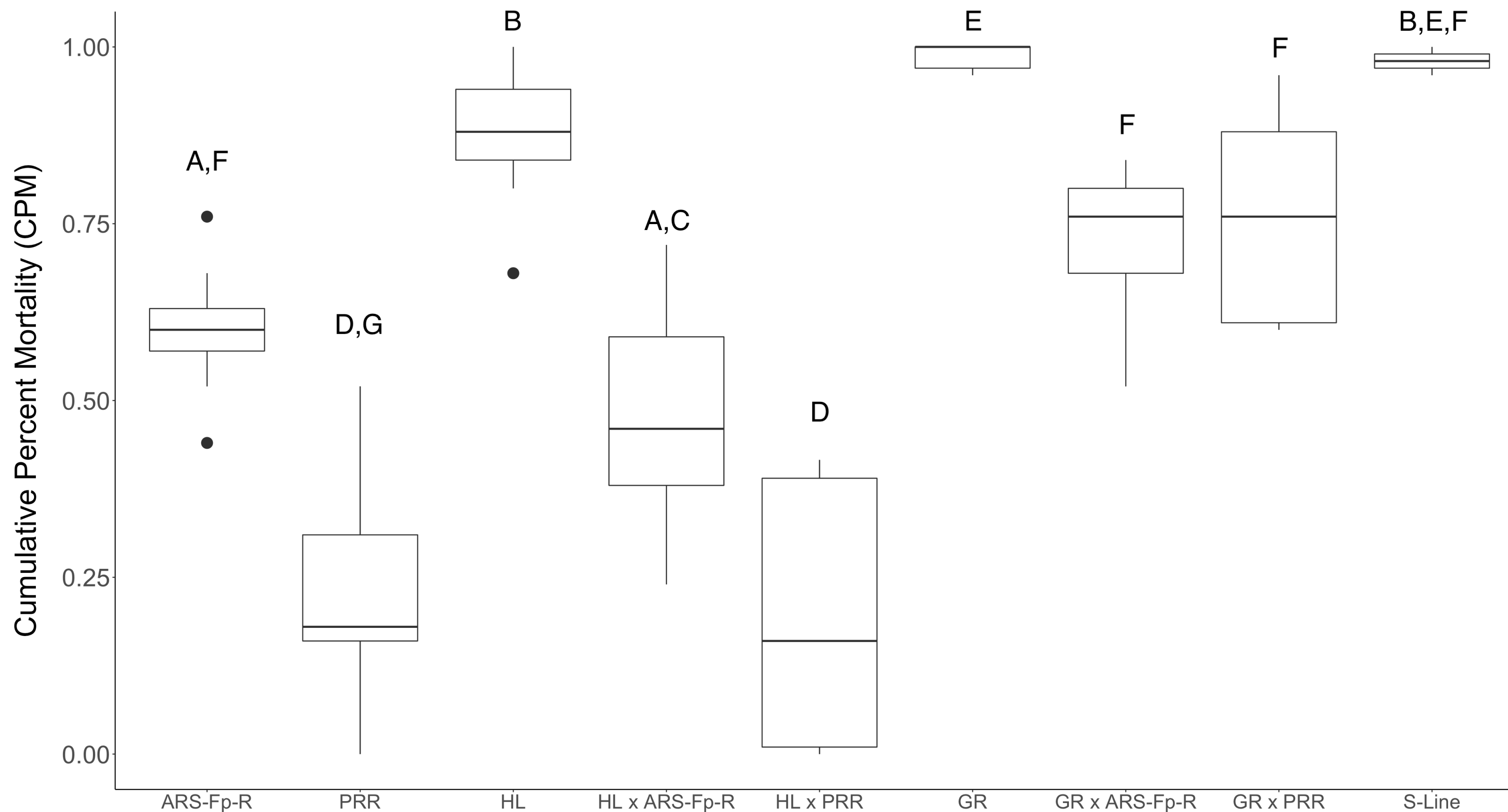

Figure 2.8. Cumulative percent mortality (CPM) by strain/cross for the fish exposed to *F. psychrophilum* only (mock injections not included) in Experiment 2. Black lines within the boxes indicate the median of the distribution. Box and whisker plots with the same letter indicate no significant differences and box and whisker plots with different letters indicate statistically significant differences.

LITERATURE CITED

Avila, B. W., D. L. Winkelman, and E. R. Fetherman. 2018. Survival of whirling-disease-resistant Rainbow Trout fry in the wild: A comparison of two strains. Journal of Aquatic Animal Health 30:280-290.

Antaya, C. 2008. Current eco-economical impacts of *Flavobacterium psychrophilum*. MMG 445 Basic Biotechnology eJournal 4:7-12.

Bandilla, M., E. T. Valtonen, L. -R. Suomalainen, P. J. Aphalo, and T. Hakalahti. 2006. A link between ectoparasite infection and susceptibility to bacterial disease in Rainbow Trout. International Journal of Parasitology 36(9):987-991.

Barnes, M. E., and M. L. Brown. 2011. A review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment. The Open Fish Science Journal 4:1-9.

Barney, P., D. E. Anderson, and P. G. Walker. 1988. Whirling disease identified in Colorado. American Fisheries Society Fish Health Section Newsletter 16:3.

Borg, A. F. 1960. Studies on myxobacteria associated with diseases in salmonid fishes. Journal of Wildlife Diseases 8:1–85.

Boulton, A. J., and A. Williford. 2018. Analyzing skewed continuous outcomes with many zeros: A tutorial for social work and youth prevention science researchers. Journal of the Society for Social Work and Research 9(4):721-740.

Branson, E. J. 1998. Rainbow Trout fry syndrome: an update. Fish Veterinary Journal 2:63-66.

Bruun, M. S., A. S. Schmidt, I. Madsen, and I. Dalsgaard. 2000. Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. Aquaculture 187:201-212.

Busch, S., I. Dalsgaard, and K. Buchmann. 2003. Concomitant exposure of Rainbow Trout fry to *Gyrodactylus derjavini* and *Flavobacterium psychrophilum*: effects on infection and mortality of host. Veterinary Parasitology 117(1-2):117-122.

Christie, M. R., M. L. Marine, R. A. French, and M. S. Blouin. 2012. Genetic adaptation to captivity can occur in a single generation. Proceedings of the Natural Academy of Science 109(1):238-242.

Christie, M. R., M. L. Marine, S. E. Fox, R. A. French, and M. S. Blouin. 2016. A single generation of domestication heritably alters the expression of hundreds of genes. Nature Communications 7(10676):1-6.

Cipriano R. C., and R. A. Holt. 2005. *Flavobacterium psychrophilum*, cause of bacterial cold-water disease and Rainbow Trout fry syndrome. United States Department of Interior, U.S. Geological Service, National Fish Health Research Laboratory. Fish Disease Leaflet No. 86.

Davis, H. S. 1946. Care and diseases of trout. Research Report, No. 12. United States Fish and Wildlife Service, Washington, D.C.

Decostere, A., E. D'Haese, M. Lammens, H. Nelis, and F. Haesebrouck. 2001. *In vivo* study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes. Journal of Fish Diseases 24:481-487.

Duan, N., W. G. Manning, C. N. Morris, and J. P. Newhouse. 1983. A comparison of alternative models for the demand for medical care. Journal of Business and Economic Statistics 1(2):115-126.

Fetherman E. R., D. L. Winkelman, G. J. Schisler, and C. A. Myrick. 2011. The effects of *Myxobolus cerebralis* on the physiological performance of whirling disease resistant and susceptible strains of Rainbow Trout. Journal of Aquatic Animal Health 23:169-177.

Fetherman, E. R., D. L. Winkelman, G. J. Schisler, and M. F. Antolin. 2012. Genetic basis of differences in myxospore count between whirling disease-resistant and -susceptible strains of Rainbow Trout. Diseases of Aquatic Organisms 102:97-106.

Fetherman, E. R., D. L. Winkelman, M. R. Baerwald, and G. J. Schisler. 2014. Survival and reproduction of *Myxobolus cerebralis*-resistant Rainbow Trout introduced to the Colorado River and increased resistance of age-0 progeny. PLoS ONE 9(5): e96954.

Fetherman, E. R., D. L. Winkelman, L. L. Bailey, G. J. Schisler, and K. Davies. 2015. Brown Trout removal effects on short-term survival and movement of *Myxobolus cerebralis*-resistant Rainbow Trout. Transactions of the American Fisheries Society 144:610-626.

FAO (Food and Agriculture Organization of the United Nations). 2020. The state of world fisheries and aquaculture. FAO, Rome.

Fraslin, C., E. Quillet, T. Rochat, N. Dechamp, J-F. Bernardet, B. Collet, D. Lallias, and P. Boudinot. 2020. Combining multiple approaches and models to dissect the genetic architecture of resistance to infections in fish. Frontiers in Genetics 11(article 677):1-20.

Groff, J. M., and S. E. LaPatra. 2001. Nutrition and fish health. *In*: C. E. Lim, and C. D. Webster, editors. Haworth Press, New York. pp. 11-78.

Hadidi, S., G. W. Glenney, T. J. Welch, J. T. Silverstein, and G. D. Wiens. 2008. Spleen size predicts resistance of Rainbow Trout to *Flavobacterium psychrophilum* challenge. The Journal of Immunology 180(6):4156-4165.

Hedrick, R. P., T. S. McDowell, G. D. Marty, G. T. Fosgate, K. Mukkatria, K. Myklebust, and M. El-Matbouli. 2003. Susceptibility of two strains of Rainbow Trout (one with suspected resistance to whirling disease) to *Myxobolus cerebralis* infection. Diseases of Aquatic Organisms 55:37-44.

Kent, M. L., J. M. Groff, J. K. Morrison, W. T. Yasutake, and R. A. Holt. 1989. Spiral swimming behavior due to cranial and vertebral lesions associated with *Cytophaga psychrophila* infections in salmonid fishes. Diseases of Aquatic Organisms 6:11-16.

Kotob, M. H., S. Menateau-Ledouble, G. Kumar, M. Abdelzaher, and M. El-Matbouli. 2016. The impact of co-infections on fish: a review. Veterinary Research 47:98.

LaFrentz, B. R., and K. D. Cain. 2004. Bacterial coldwater disease. An Extension Bulletin for the Western Regional Aquaculture Center. University of Idaho, Moscow.

Leeds, T. D., J. T. Silverstein, G. M. Weber, R. L. Vallejo, Y. Palti, C. E. Rexroad III, J. Evenhuis, S. Hadidi, T. J. Welch, and G. D. Wiens. 2010. Response to selection for bacterial cold water disease resistance in Rainbow Trout. Journal of Animal Science 88(6):1936-1946.

Lhorente, J. P., J. A. Gallardo, B. Villanueva, M. J. Carabano, and R. Neira. 2014. Disease resistance in Atlantic Salmon (*Salmo salar*): Coinfection of the intracellular bacterial pathogen *Piscirickettsia salmonis* and the Sea Louse *Caligus rogercresseyi*. PLoS ONE 9(4):e95397.

Lumsden, J. S., K. Young, K. Welsh, J. MacInnes, S. Russell, and S. Hesami, 2006. Management approaches for coldwater disease caused by *Flavobacterium psychrophilum*. Proceedings of the Canadian Freshwater Aquaculture Symposium-Aquaculture Canada. Aquaculture Association of Canada Special Publication No. 11:111-117. St. Andrews, New Brunswick.

Ma, J., T. J. Bruce, L. P. Oliver, and K. D. Cain. 2019. Co-infection of Rainbow Trout (*Oncorhynchus mykiss*) with infectious hematopoietic necrosis virus and *Flavobacterium psychrophilum*. Journal of Fish Diseases 42:1065-1076.

Markiw, M. E., and K. Wolf. 1974a. *Myxosoma cerebralis:* isolation and concentration from fish skeletal elements - sequential enzymatic digestions and purification by differential centrifugation. Journal of the Fisheries Research Board of Canada 31:15-20.

Markiw, M. E., and K. Wolf. 1974b. *Myxosoma cerebralis:* comparative sensitivity of spore detection methods. Journal of the Fisheries Research Board of Canada 31:1597-1600.

Martinez, J. L., A. Casado, and R. Enriquez. 2004. Experimental infection of *Flavobacterium psycrhophilum* in fins of Atlantic Salmon *Salmo salar* revealed by scanning electron microscopy. Diseases of Aquatic Organisms 59:74-84.

Michel, C., D. Antonio, and R. P. Hedrick. 1999. Production of viable cultures of *Flavobacterium psychrophilum*: approach and control. Research in Microbiology 150:351-358.

Miller, M. P., and E. R. Vincent. 2008. Rapid natural selection for resistance to and introduced parasite on Rainbow Trout. Evolutionary Applications 1:336-341.

Nehring, R. B., and K. G. Thompson. 2001. Impact assessment of some physical and biological factors in the whirling disease epizootic among wild trout in Colorado. Colorado Division of Wildlife Special Report 76.

Nehring, R. B., and K. G. Thompson. 2003. Whirling disease risk assessment: the Colorado perspective. In: Managing the risk. Proceedings of the 9th annual whirling disease symposium. Whirling Disease Initiative, Bozeman, MT, p. 31-32.

Nicolas, P., S. Mondot, F. Achaz, C. Couchenot, J. Bernardet, and E. Duchaud. 2008. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Applied and Environmental Microbiology 74:3702-3709.

Nilsen, H., A. B. Olsen, Ø. Vaagnes, H. Hellberg, K. Bottolfsen, H. Skjelstad, and D. J. Colquhoun. 2011. Systemic *Flavobacterium psychrophilum* infection in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. Journal of Fish Diseases 34(5):403-408.

O'Grodnick, J. J. 1975. Whirling disease *Myxosoma cerebralis* spore concentration using the continuous plankton centrifuge. Journal of Wildlife Diseases 11:54-57.

Ostland, V. E., D. G. McGrogan, and H. W. Ferguson. 1997. *Cephalic osteochondritis* and necrotic scleritis in intensively reared salmonids associated with *Flexibacter psychrophilus*. Journal of Fish Diseases 20:443-451.

Post, G. P. 1987. Textbook of Fish Health. T.F.H Publications, New Jersey.

Schisler, G. J., K. A. Myklebust, and R. P. Hedrick. 2006. Inheritance of *Myxobolus cerebralis* resistance among F1-generation crosses of whirling disease resistant and susceptible Rainbow Trout strains. Journal of Aquatic Animal Health 18:109-115.

Schisler, G. J., and E. R. Fetherman. 2009. Resistant Rainbow Trout in Colorado: Current status and uses. Colorado Division of Wildlife, Aquatic Wildlife Research Section. Fort Collins, Colorado.

Schisler, G. J., E. R. Fetherman, and B. Neuschwanger. 2011. Salmonid Disease Studies. Federal Aid Project F-394R-10. Federal Aid in Fish and Wildlife Restoration, FINAL Progress Report. Colorado Division of Wildlife, Aquatic Wildlife Research section. Fort Collins, Colorado.

Schrag, S. J., and P. Wiener. 1995. Emerging infectious disease: what are the relative roles of ecology and evolution? Trends in Ecology and Evolution 10(8):319-324.

Silverstein, J. T., R. L. Vallejo, Y. Palti, T. D. Leeds, C. E. Rexroad III, T. J. Welch, G. D. Wiens, and V. Ducrocq. 2009. Rainbow Trout resistance to bacterial cold-water disease is moderately heritable and is not adversely correlated with growth. Journal of Animal Science 87:860-867.

Starliper, C. E., 2011. Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. Journal of Advanced Research 2:97-108.

Sudheesh, P. S., and K. D. Cain. 2016. Optimization of efficacy of a live attenuated *Flavobacterium psychrophilum* immersion vaccine. Fish and Shellfish Immunology 56:169-180.

USDA (U. S. Department of Agriculture). 2016. Trout Production. USDA. ISSN: 1949-1948. Washington D.C.

USDA (U. S. Department of Agriculture). 2017. Trout Production. USDA. ISSN: 1949-1948. Washington D.C.

USDA (U. S. Department of Agriculture). 2018. Trout Production. USDA. ISSN: 1949-1948. Washington D.C.

USDA (U. S. Department of Agriculture). 2019. Trout Production. USDA. ISSN: 1949-1948. Washington D.C.

USDA (U. S. Department of Agriculture). 2020. Trout Production. USDA. ISSN: 1949-1948. Washington D.C.

Wagner, E. J., C. Wilson, R. Arndt, P. Goddard, M. Miller, A. Hodgson, R. Vincent, and K. Mock. 2006. Evaluation of disease resistance of the fish Lake-DeSmet, Wounded Man, and Harrison Lake strains of Rainbow Trout exposed to *Myxobolus cerebralis*. Journal of Aquatic Animal Health 18:128-135.

Wiens, G. D., S. E. LaPatra, T. J. Welch, J. P. Evenhuis, C. E. Rexroad III, and T. D. Leeds. 2013. On-farm performance of Rainbow Trout (*Oncorhynchus mykiss*) selectively bred for resistance to bacterial cold water disease: Effect of rearing environment on survival phenotype. Aquaculture (388-391):128-136.

Wood, J. W. 1974. Diseases of Pacific Salmon, their prevention and treatment, 2nd edition. Washington Department of Fisheries, Olympia.

CHAPTER 3: HATCHERY REARING PRACTICES AFFECT POST-STOCKING SURVIVAL

INTRODUCTION

Aquaculture is an important aquatic production resource worldwide (FAO 2020). In 2019, the United States produced just over 169 million tons of trout for food and stocking worth more than $230 million US dollars (USDA 2020). Of the 169 million tons of trout produced, 72% were produced for restoration and conservation and stocking for angling opportunities. Within Colorado, USA recreational fishing supplies $2.4 billion US dollars into the economy, supports tens of thousands of jobs, and provides over $750 million US dollars in salary and wages (Southwick Associates 2017). Clearly trout are of great recreational and economic value.

A hatchery environment is drastically different from a natural environment and research suggests that fish raised in the hatchery and stocked into the wild have lower future survival and/or reproductive effort/success compared to wild fish (Snyder et al. 1996; Araki et al. 2007; Araki et al. 2008; Frankham 2008; Williamson et al. 2010). Further, research shows that fitness declines can be attributed to relaxed natural selection, environmental effects of the hatchery, inbreeding, and domestication (Lynch and O'Hely 2001; Araki et al. 2008; Charlesworth and Willis 2009; Hansen and Mensberg 2009; Williamson et al. 2010). The hatchery environment has been shown to alter genes and decrease brain size which may affect predator avoidance in hatchery trout (Marchetti and Nevitt 2003; Christie et al. 2012, 2016). Hatchery practices used to meet production demands, such as high rearing densities or use of lower-cost, potentially lower-quality, feeds may also affect fish when stocked into the wild.

It is common practice to rear trout at high densities to maximize the number of fish available to mitigate the large demand for recreational fishing (Aprahamian et al. 2003). Maximizing production by rearing fish at high densities can impact important biological factors, such as mortality, food conversion, growth rate, fin condition, and disease. Density has been shown to have both positive and negative effects on mortality in a hatchery setting (Ellis et al. 2002); however, there is extensive support for the idea that mortality increases with higher rearing densities (e.g., Kincaid et al. 1976; Holm et al. 1990;

Kindschi et al. 1991; Pickering et al. 1991; Winfree et al. 1998; Sirakov and Ivancheva 2008). Studies also suggest a negative effect on food conversion (i.e., increased food conversion ratio) with increased rearing density (Kincaid et al. 1976; Atay et al. 1988; Kindschi et al. 1991; Logan and Johnston 1992). Within experiments, growth (length or weight of fish at the end of a time period) is a common metric to monitor, and many studies have suggested that increasing rearing density reduces growth (Kilambi et al. 1977; Leatherland and Cho 1985; Holm et al. 1990; Unlu and Baran 1992; Alanärä and Brännäs 1996; Sirakov and Ivancheva 2008; Kavanagh and Olsen 2014; Rosengren et al. 2017). High rearing densities have also been shown to reduce fin condition (Boydstun and Hopelain 1977; Makinen and Ruohonen 1990; Purser and Hart 1991; Miller et al. 1995; Wagner et al. 1997; Winfree et al. 1998) which may affect survival after stocking (Nicola and Cordone 1973) and may lead to bacterial infections (Wagner et al. 1997). One of the most important bacterial pathogens of trout in an aquaculture setting is *Flavobacterium psychrophilum*, the causative agent of Bacterial Coldwater Disease (BCWD), due to the extensive mortality that can occur due to infection (Michel et al. 1999). A suggested preventative measure to reduce BCWD outbreaks is to reduce rearing densities (Cipriano and Holt 2005; Starliper 2011). Reducing rearing density within the hatchery has also been shown to improve post-stocking growth, social dominance, and survival of Brown Trout *Salmo trutta* (Brockmark and Johnsson 2010).

The hatchery environment and rearing practices affect fish biologically in the hatchery as well as demographically post-stocking. Understanding how hatchery rearing practices affect populations post-stocking is important to maximize the quality, quantity, and economic value of fish stocked for conservation and recreational purposes. The overall goal for my study was to determine, experimentally, if rearing density and feed affect post-stocking survival of Rainbow Trout *Oncorhynchus mykiss* fry when stocked into a put-grow-and-take fishery.

METHODS

The hatchery-rearing portion of the experiment, completed at the Colorado Parks and Wildlife (CPW) Bellvue Fish Research Hatchery, consisted of two rearing densities and two diets, resulting in four different rearing conditions that each had two replicates (Figure 3.1). Fish were reared at either a low density (maximum rearing index of 0.5) or high density (maximum rearing index of 2.0). Additionally, the basic feeds from two commercial companies, Bio Oregon and Rangen, were evaluated to determine if feed type used by CPW had an influence on post-stocking survival.

I used German Rainbow x Harrison Lake (hereafter, GR x HL) Rainbow Trout for this experiment because they are one of the most common strains produced in CPW hatcheries (Eric Fetherman, personal communication). HxH eggs were spawned at the CPW Bellvue Fish Research Hatchery. Eggs were held in egg cups, short PVC pipes with fine-mesh screen bottoms and a slow flow stream directed in the center to allow for constant aeration, in two experimental tanks, one for each diet treatment, and dead eggs were removed daily to prevent fungal growth. Upon hatching, fish were placed into experimental tanks and deformed and erratically swimming fish were removed through the swim-up phase (roughly three weeks).

At two weeks post-swim-up, fish were counted and distributed into randomly assigned fiberglass hatchery troughs (Figure 3.1). Density was manipulated using a common rearing index calculated in pounds of fish per cubic foot of water that is no greater than one-half the average fish length in inches. Piper et al. (1982) recommends a rearing index of 0.5, which resulted in 1,000 fish per trough in the low-density treatment. Colorado Parks and Wildlife hatcheries typically maintain fish at a rearing index of 2.0 and I used this as my high-density treatment, resulting in 4,000 fish per trough. The volume for each trough was manipulated throughout the three-month experiment to keep the rearing density indices from exceeding 0.5 or 2.0. As fish grew, tank volumes changed three times: 1) 2.7 cubic feet, 2) 5.4 cubic feet, and 3) 10.8 cubic feet. Upon volume change, density index was reduced to nearly half of the maximum, and fish were allowed to grow up to the maximum before the volume was changed again.

An initial batch weight was taken from each trough and used to assign a feeding rate (% BW/d) using the recommended feeding rates for each company (Fetherman et al. 2019; Appendix 3.1). Batch weights were taken weekly and feeding rate was adjusted based on these weights. Feeding occurred six to eight times daily over three months. Troughs were treated independently, and once a given trough reached the maximum average individual weight recommended for a given feed size, the fish were switched to the next feed size. Flows were adjusted as fish grew to maintain appropriate dissolved oxygen concentrations and water exchange.

After the three-month rearing period, twenty fish from each trough were euthanized and weight, length, and liver weight for each fish were recorded. Liver weight was used to calculate hepatosomatic index (HSI) for each fish to determine the percent weight the liver contributed to the total body weight (Fetherman et al. 2019). To allow for post-stocking survival analysis, fish were adipose clipped and tagged in the intraperitoneal cavity using 12-mm Passive Integrated Transponder (PIT) tags. PIT tags provide individual identification which allows for better survival estimation compared to batch marks. Fish ranged between 95 and 106 mm total length and 11 and 14 g in weight at the time of stocking. Equal numbers of fish from each density and feed treatment were tagged, with 710-725 fish being tagged from each hatchery trough, resulting in 1,430-1,450 fish tagged per each density by feed treatment type (Table 3.1). All fish (20,000), tagged (2,899 high-density and 2,869 low-density fish), and untagged extras (13,101 high-density and 1,131 low-density fish), were stocked into Parvin Lake (Red Feather Lakes, Colorado) in September 2017.

Parvin Lake, 2,479 m elevation and 24.78 hectares, is located 45 miles northwest of Fort Collins, Colorado and houses the CPW Parvin Lake Research Station. The reservoir is stocked annually with fingerling Brown Trout, Rainbow Trout, and Splake *Salvelinus namaycush x S. fontinalis*. Tiger Muskies *Esox masquinongy x E. lucius* were stocked in 2000 through 2003 to manage the large White Sucker *Catostomus commersonii* population. The largest threat to survival of stocked fish is predation from larger fish and double-crested cormorants *Phalacrocorax auritus*, osprey *Pandionidae haliaetus*, and great blue herons *Ardea herodias*.

Recaptures were conducted using a boat-mounted electrofishing unit every two weeks between September and November 2017 (four sampling occasions), and one time per month in April, June, and September 2018. Shocking events were conducted at night, by following the shoreline clockwise, sampling the entire lake to account for Rainbow Trout distribution throughout the lake. The same methods and route were used at each recapture event. At each recapture event, all Rainbow Trout captured were scanned for PIT tags. Lengths and weights of recaptured fish were taken at two-, seven-, and twelve-months post-stocking to allow for a comparison of post-stocking conditions among treatments. A maximum of five fish per treatment were euthanized at two- and seven-months post-stocking to compare HSI values among treatments. Fish were not sacrificed at twelve-months post-stocking due to low and uneven numbers of recaptures.

Statistical analysis pre-stocking

Comparisons of fish condition, length, weight, and HSI pre-stocking were conducted using Bayesian regression using Just Another Gibbs Sampler (JAGS) within program R (version 4.03). For example, for HSI:

$$\mathrm{g}(\mathrm{S}_0, S_1, S_2, S_3) = \mathrm{S}_0 + \mathrm{S}_1\mathrm{Density}_i + \mathrm{S}_2\mathrm{Feed}_i + \mathrm{S}_3(\mathrm{Density}_i * \mathrm{Feed}_i)$$

$$\begin{gathered}[\mathrm{S}_0, S_1, S_2, S_3, \sigma \mid \mathbf{y}] \propto \textstyle\prod_{i=1}^{n} \ \mathrm{normal}(y_i \mid g(S_0, S_1, S_2, S_3), \sigma) \\ \times\mathrm{uniform}(\sigma \mid 0, 100000) \\ \times\mathrm{gamma}(S_0 \mid 2, 1) \\ \times\mathrm{uniform}(S_1 \mid -1000, 1000) \\ \times\mathrm{uniform}(S_2 \mid -1000, 1000) \\ \times\mathrm{uniform}(S_3 \mid -1000, 1000)\end{gathered}$$

Because fish were the same age and raised in the same hatchery facility, Fulton's condition factor (Anderson and Neumann 1996) was used in all condition comparisons. The data (yi) consisted of Fulton's condition factor (Appendix 3.2), length (Appendix 3.3), weight (Appendix 3.4), or HSI (Appendix 3.5) for each hatchery trough. The parameters within condition, length, weight, and HSI means models were the mean for each parameter (S0, mean of the response at the mean value of the covariates), the change in the mean due to rearing density (S1), the change in the mean due to feed type

(S2), the change in the mean due to a density by feed interaction (S3), and the standard deviation (σ) of the observations.

Vague prior information was used for the standard deviation of the observations taken from a uniform distribution ranging between zero and 100,000. Vague prior information for condition was also used for the parameters that represent the changes in the mean (S1, S2, and S3) with a mean of zero and a variance of 1,000 from a normal distribution, and for length, weight, and HSI from a uniform distribution with a range between -1,000 and 1,000. Semi-informative prior information was used for the mean condition and HSI (S0) was taken from a gamma distribution with a shape equal to two and a rate equal to one; this was modified to provide a wider distribution from the data presented in Fetherman et al. (2019). Weakly-informative prior information was used for the mean length and weight (S0) taken from a gamma distribution with a shape equal to 100 (length) or ten (weight) and a rate equal to one which was based off of prior biological knowledge of Rainbow Trout fry (unpublished data). Posterior inference for model parameters and derived quantities were based on retaining 1,000,000 (condition, length, and weight) or 100,000 (HSI) MCMC samples following a burn-in of 20,000 samples.

Visual inspection of trace plots for model parameters and the use of Gelman and Rubin diagnostics (Gelman and Rubin 1992) equal to one indicated that the MCMC chains mixed and converged to the target distributions. Gelman and Rubin diagnostic values less than 1.1 indicate model convergence (Hobbs and Hooten 2015). Posterior predictive checks also known as Bayesian p-values were used, looking at the mean and standard deviation of the data (Table 2), to determine lack of fit. Bayesian p-values less than 0.1 and greater than 0.9 indicate lack of fit (Hobbs and Hooten 2015). Posterior distributions of model parameters were examined and used to present the derived quantities for mean condition, length, weight, or HSI and the associated 95% credible intervals for each treatment (Bio Oregon low rearing density; Bio Oregon high rearing density; Rangen low rearing density; Rangen high rearing density).

Statistical analysis post-stocking

Comparisons of fish condition and HSI post-stocking was conducted using Bayesian regression using Just Another Gibbs Sampler (JAGS) within program R (version 4.03).

$$\mathrm{g}(\mathrm{S}_0, S_1, S_2, S_3) = \mathrm{S}_0 + \mathrm{S}_1\mathrm{Density}_i + \mathrm{S}_2\mathrm{Feed}_i + \mathrm{S}_3(\mathrm{Density}_i * \mathrm{Feed}_i)$$

$$\begin{gathered}[\mathrm{S}_0, S_1, S_2, S_3, \sigma \mid \mathbf{y}] \propto \textstyle\prod_{i=1}^{n} \ \mathrm{normal}(y_i \mid g(S_0, S_1, S_2, S_3), \sigma) \\ \times \mathrm{uniform}(\sigma \mid 0, 100000) \\ \times \mathrm{gamma}(S_0 \mid 2, 1) \\ \times \mathrm{uniform}(S_1 \mid -1000, 1000) \\ \times \mathrm{uniform}(S_2 \mid -1000, 1000) \\ \times \mathrm{uniform}(S_3 \mid -1000, 1000)\end{gathered}$$

The data (y_i) consisted of Fulton's condition factor from recaptured fish calculated for hatchery troughs at two-, seven- and twelve-months post-stocking (Appendix 3.2), and HSI from as many as five recaptured fish for each hatchery trough at two- and seven-months post-stocking (Appendix 3.5). Due to only six fish being recaptured in September 2018, HSI was not taken at twelve-months post-stocking. The parameters, derived quantities, prior information, and MCMC samples used within the post-stocking means model was the same as the pre-stocking condition or HSI section. Posterior inference for the two-, seven- and twelve-months post-stocking condition and two- and seven-months post stocking HSI model parameters and derived quantities were based on 100,000 MCMC samples following convergence after 20% of an iteration burn-in period.

Survival analysis was conducted using a capture-recapture Cormack-Jolly-Seber hierarchical Bayesian model (Royle and Dorazio 2008; Appendix 3.6),

$$(\phi_i) = \text{inverse logit}(\beta_0 + \beta_{\text{Density}} * (\text{Density}_i) + \beta_{\text{Feed}} * (\text{Feed}_i) + \beta_{\text{Density*Feed}} * (\text{Density}_i * \text{Feed}_i))$$

$$\begin{gathered}
[\text{z}, \beta_0, \beta_{\text{Density}}, \beta_{\text{Feed}}, \beta_{\text{Density*Feed}}, \boldsymbol{p} \mid \mathbf{y}] \propto \prod_{i=1}^{n} \prod_{t=2}^{8} \text{ Bernoulli}(y_{it} \mid p_t * z_{it}) \\
\times \prod_{t \in 2,3,4,5} \text{Bernoulli}(z_{it} \mid z_{i(t-1)}, \phi_i^{\frac{2}{52}}) \\
\times \prod_{t \in 6} \text{Bernoulli}(z_{it} \mid z_{i(t-1)}, \phi_i^{\frac{22}{52}}) \\
\times \prod_{t \in 7} \text{Bernoullli}(z_{it} \mid z_{i(t-1)}, \phi_i^{\frac{12}{52}}) \\
\times \prod_{t \in 8} \text{Bernoulli}(z_{it} \mid z_{i(t-1)}, \phi_i^{\frac{10}{52}}) \\
\times \text{beta}(p_i \mid 1, 1) \\
\times \text{normal}(\beta_0 \mid 0, 1.96) \\
\times \text{normal}(\beta_{\text{Density}} \mid 0, 1.96) \\
\times \text{normal}(\beta_{\text{Feed}} \mid 0, 1.96) \\
\times \text{normal}(\beta_{\text{Density*Feed}} \mid 0, 1.96)
\end{gathered}$$

using Just Another Gibbs Sampler (JAGS) within program R (version 4.03). The data consisted of eight total encounters ($t = 8$) for each PIT tagged fish, where the first encounter is represented by the stocking event where all fish were alive, and the following time periods are the seven sampling occasions. Encounter histories for each fish were used to calculate model parameters (ρ_t, detection probability varied by time; β_0, intercept; β_{Density}, effect of density; β_{Feed}, effect of feed; $\beta_{\text{Density*Feed}}$, effect of the interaction between density and feed) and the derived quantities (Φ, 12-month interval survival; $\Phi^{8/52}$ two-month survival; $\Phi^{22/52}$ seven-month survival for the densities and feeds). Vague prior information was used for the beta parameters in the model with means equal to zero and the variance equal to 1.96 from a normal probability distribution. Posterior inference for model parameters and derived quantities were based on 250,000 MCMC samples following convergence after discarding 25,000 samples as burn-in.

Visual inspection of condition and HSI model's trace plots for model parameters and the use of Gelman and Rubin diagnostics (Gelman and Rubin 1992) equal to one indicated that the MCMC chains mixed and converged to the target distributions. Visual inspection of trace plots for survival model parameters and the use of the Heidelberger and Welch diagnostic (Heidelberger and Welch 1983) indicated that the MCMC chain mixed and converged to the target distributions. Bayesian p-values were used, looking at the mean of the data and standard deviation of the data (Table 2), to determine lack of fit.

Posterior distributions of each model parameter (two-, seven-, and twelve-months post stocking condition; two- and seven-months post stocking HSI) were examined and used to present the derived quantities for mean condition or HSI and the associated 95% credible intervals (equal tailed) for each treatment at each time period (Bio Oregon low rearing density; Bio Oregon high rearing density; Rangen low rearing density; Rangen high rearing density). Posterior distributions were examined and are presented for detection probability, effects of rearing density, feed type, the interaction between rearing density and feed type, twelve-month interval survival, survival after two-months and seven-months, and estimated number of fish (survival probability*number of fish stocked).

RESULTS

Pre-stocking

During hatchery rearing, the total amount of feed used for each trough ranged between 5,816 g and 31,000 g (Table 3.3). The average amount of feed used for low-density fish was 6,801 g ± 794 (SD) and the average amount of feed use for high-density fish was 26,937 g ± 4,334 (SD). At the end of the experiment, the average condition factor for each treatment ranged between 1.07 and 1.15 (SD = 0.04; Figure 3.2). The mean length of fish for each treatment ranged between 95.7 and 105.8 mm (SD = 6.32; Figure 3.3). The mean weight of fish for each treatment ranged between 10.6 and 14.2 g (SD = 2.43; Figure 3.4). No differences in condition factor, length, or weight were observed due to the large overlap in 95% credible intervals. The mean HSI of fish for each treatment ranged between 1.43 and 2.82 (SD = 0.33). Fish reared at a low density and fed Bio Oregon feed in the hatchery had the highest HSI value at the time of stocking and the highest HSI value among all time periods observed (Figure 3.5).

Post-stocking

Mean condition factors for the treatments ranged between 1.14 and 1.18 (SD = 0.06) at two-months post-stocking, between 0.96 and 1.00 (SD = 0.02) at seven-months post-stocking, and between 1.07 and 1.09 (SD = 0.12) at twelve-months post-stocking. There were no differences among the treatments within the two-, seven-, or twelve-month periods (Figure 3.2). However, on average, fish at seven-months post-stocking, which represents the time period just after winter, showed lower condition

compared to those at two-months post-stocking, which was expected. Mean HSI values for the treatments ranged between 1.38 and 1.52 (SD = 0.12) at two-months post-stocking, and between 1.09 and 1.41 (SD = 0.12) at seven-months post-stocking. There were no differences in HSI values among treatments within each time period nor at two- or seven-months post-stocking (Figure 3.5).

More fish from the low-density treatments were captured over the course of the experiment than were fish from the high-density treatments, but the number of fish captured that had been reared on the Bio Oregon versus Rangen feeds were similar (Table 3.1). Detection probability ranged between 0.025 and 0.18 among the seven encounter occasions (Figure 3.6). Density was the only factor that affected post-stocking survival (Figure 3.7). The twelve-month interval apparent survival (Φ) for each density ranged between 0.5% and 2.5% (Figure 8). At two-, seven-, and twelve-months post-stocking, fish reared at a lower density had higher apparent survival compared to fish reared at a higher density (Figures 3.8, 3.9, 3.10). The estimated number of fish remaining within the system after two-months was higher for the high-density fish compared to the low-density fish (6,596 and 2,158, respectively), but was higher for the low-density fish compared to the high-density fish at twelve-months post-stocking (99 and 81, respectively), although there is large overlap of the twelve month 95% credible intervals (Figure 3.11).

DISCUSSION

Rearing density affected post-stocking apparent survival and was higher for low rearing density treatments at all time points post-stocking. Due to lower apparent survival of fish reared at high densities, similar numbers of fish in low- and high-density rearing treatments survived to twelve-months post-stocking despite four times as many fish being stocked from the high-density treatments. Fish condition, length, and weight did not differ at the time of stocking, however, HSI values were higher for the fish reared at low density and fed with Bio Oregon at the time of stocking. Feed effects were not observed for condition, HSI, or survival at any time post-stocking.

My study revealed that rearing fish at a lower density in the hatchery resulted in higher post-stocking survival for each of the time periods examined. Traditionally, fish are reared at higher densities for stocking into wild systems in hopes of maximizing the number of fish available to anglers

(Aprahamian et al. 2003). However, there may be advantages to rearing fish at lower density, such as increased growth, lower probability of disease transmission in the hatchery, decreased time in finding food post-stocking and increased survival post-stocking (Procarione et al. 1999; Brockmark et al. 2010; Pulkkinen et al. 2010; Barnes et al. 2013). Brockmark et al. (2010) and Brockmark and Johnson (2010) showed that lower rearing-densities promote higher post-stocking survival for Brown Trout, concluding that “less is more”. Higher smolt-to-adult survival has also been reported in Chinook Salmon *O. tshawytscha* and Coho Salmon *O. kisutch* reared at lower densities (Ewing and Ewing 1995; Olson and Paiya 2013). Increased survival is essential in put-grow-and-take fisheries such as Parvin Lake, and could result in more fish reaching catchable size and becoming available to anglers.

Increased survival due to lower rearing density may not compensate for the reduced number of fish produced in all situations. It may be advantageous to maximize stocking density within a system, such as a put-and-take fishery, even if it means rearing fish at higher densities to do so. The effects on survival due to high-rearing densities would be less important in a put-and-take fishery when fish do not need to exhibit high long-term survival. However, there are drawbacks to raising fish at high densities. Fish reared at high densities have been shown to have lower dominance status, variable weight, length and condition, increased mortality and increased stress, all of which can lead to increased susceptibility to pathogen infection (Fagerlund et al. 1981; Iguchi et al. 2003; Brockmark and Johnsson 2010). Raising fish at higher densities may lead to increased stress and damage to the skin which could lead to BCWD outbreaks (LaFrentz and Cain 2004; Barnes and Brown 2011). Although a BCWD outbreak did not occur in the hatchery during this experiment, higher density rearing conditions might favor increased transmission, and mortality associated with BCWD outbreaks can range from 5% to 90% (Nilsen et al. 2011; Barnes and Brown 2011). In Idaho, hatchery mortality due to BCWD commonly varies around 30% (Dr. Kenneth Cain, personal communication). If a BCWD outbreak did occur, mortalities could be similar to other studies and the resulting number of fish left to stock would have been drastically reduced, thereby eliminating the advantage of raising fish at higher density. Additionally, BCWD outbreaks are usually controlled by antibiotic treatment that could also reduce growth.

Another disadvantage of rearing fish at high densities is the physical and financial resources required to maintain the larger number of fish. Higher numbers of fish require more food and increased water resources, or antibiotics due to disease outbreaks. In my experiment, the high-density treatments required about four times the amount time for cleaning and about four times the amount of feed. The increased expenditure of resources did not result in more fish at 12 months post-stocking and there were about 18% fewer fish surviving after twelve-months post stocking. The difference in the number of fish remaining from the density treatments at twelve-months post-stocking was not large or significant, suggesting no benefit of rearing fish at high-densities when annual survival is required. Therefore, my study suggests that increased resources needed to rear fish at high densities may not be worth the additional costs if roughly the same number of fish reach a size at which they are available to anglers.

At the request of CPW, we stocked all fish from each tank, which resulted in 16,000 fish being stocked from the high-density treatments and 4,000 from the low-density treatments. Ideally, we would have stocked equal numbers from each treatment to minimize the possibility that stocking density would affect post-stocking survival and confound our analysis. It is possible that our stocking density influenced our results (Kelly-Quinn and Bracken 1989); however, condition factor was relativity good at each evaluation period and did not differ among densities, indicating that fish were obtaining enough natural food and had not exceeded the carrying capacity of salmonid fingerlings in Parvin Lake. However, another source of mortality could be predation. Stocking higher numbers of fish may actually increase predation due to predators having more contact with stocked fish or providing a consistent search image and choosing to capitalize on the availability of the stocked fish.

It is common for rearing density and diet to affect growth and condition in hatcheries (Procarione et al. 1999; Brockmark and Johnsson 2010; Olson and Paiya 2013) but less is known about the effects of rearing conditions on growth and condition of fish post-stocking. Fish within this experiment showed no difference in condition, length or weight at the time of stocking however, HSI was higher for the fish reared at low-density and fed Bio Oregon feed compared to other treatments. Higher HSI suggests more fat content available to fish compared to lower HSI values. Fish with higher HSI values may exhibit

different behaviors, requiring less bold movements when foraging post-stocking. Since hatchery-reared fish are considered naive in relation to predators and feeding (Suboski and Templeton 1989; Brown and Laland 2001; Einum and Fleming 2001; Brown et al. 2003) density and feed may be important considerations when stocking fish into systems where predators are present. Fish with higher HSI values may be able to hide from predators or prevent dangerous behaviors like finding new food sources if they are able to survive longer on their own resources.

My study suggests that rearing density affects fingerling Rainbow Trout post-stocking survival. Higher numbers of fish stocked due to fish being reared at higher densities within the hatchery did not result in more total fish at twelve months compared to the low-density treatment. Raising fish at high density uses increased resources and may not provide any additional catchable fish for anglers. Additionally, other factors such as disease and predation may be considerations for making decisions about rearing and stocking density.

Table 3.1. Number of tagged fish stocked per treatment (low or high density; Bio Oregon or Rangen feed) and associated capture and recapture numbers ([ ]) at two-, seven-, and twelve-months post-stocking. Within the 2-month time period there are four sampling occasions.

Treatment	# Fish Stocked	2-Months	7-Months	12-Months
Low, Bio Oregon	1430	45 [10]	27 [6]	1 [1]
High, Bio Oregon	1449	20 [4]	7 [0]	1 [0]
Low, Rangen	1439	39 [6]	25 [3]	3 [1]
High, Rangen	1450	36 [4]	11 [3]	1 [0]

Table 3.2. Bayesian p-values for the mean of the data (mean) and the standard deviation of the data (SD) for each model and period.

Period	Model	p-value (mean)	p-value (SD)
Pre-stocking	Condition	0.5	0.79
Pre-stocking	Length	0.48	0.8
Pre-stocking	Weight	0.46	0.83
Pre-stocking	HSI	0.49	0.67
Post-stocking 2-months	Condition	0.5	0.58
Post-stocking 7-months	Condition	0.5	0.57
Post-stocking 12-months	Condition	0.5	0.62
Post-stocking 2-months	HSI	0.5	0.83
Post-stocking 7-months	HSI	0.5	0.73
Post-stocking	Survival	0.57	0.57

Table 3.3. Total amount of feed (g) used for each tank, density, and feed type during the hatchery rearing portion of the experiment.

Tank	Density	Feed	Total Feed (g)
7	Low	Bio Oregon	5,816
8	High	Bio Oregon	24,155
9	Low	Rangen	7l,716
10	High	Rangen	31,001
17	High	Rangen	30,253
18	Low	Rangen	7,042
19	Low	Bio Oregon	6,634
20	High	Bio Oregon	22,343

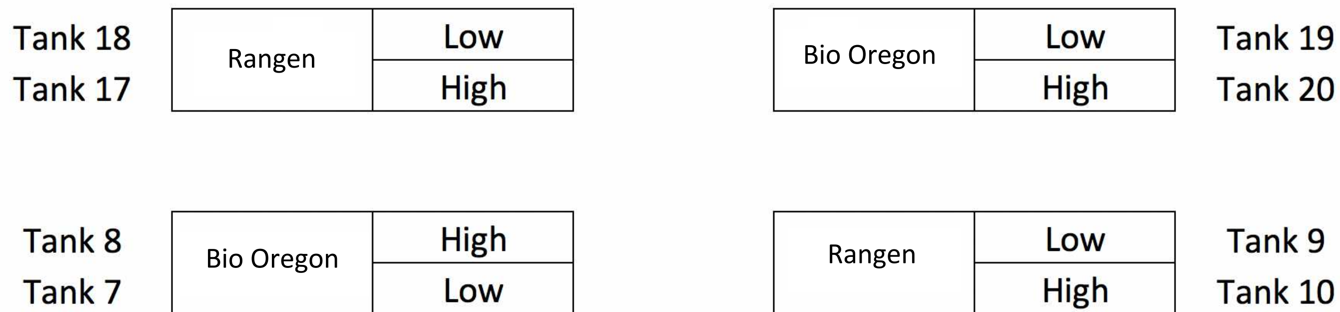

Figure 3.1. Paired experimental hatchery troughs used to rear the Rainbow Trout (H×H) at two densities (low and high) and on two feeds (Bio Oregon and Rangen) at the Colorado Parks and Wildlife Bellvue Fish Research Hatchery.

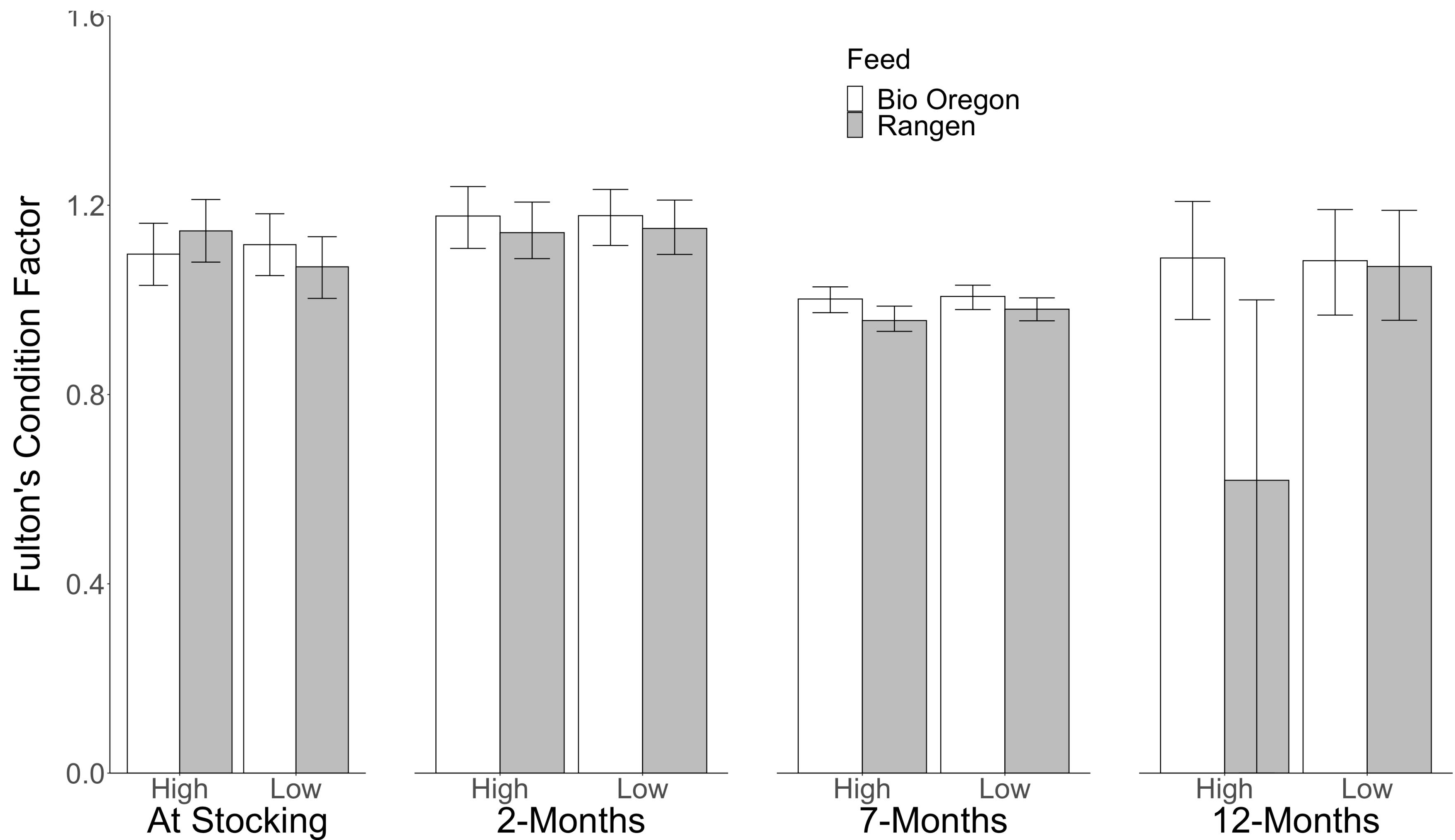

Figure 3.2. Mean condition (Fulton's condition factor; 95% credible interval bars) at the time of stocking, and two-, seven-, and twelve-months post-stocking for each rearing density and feed type.

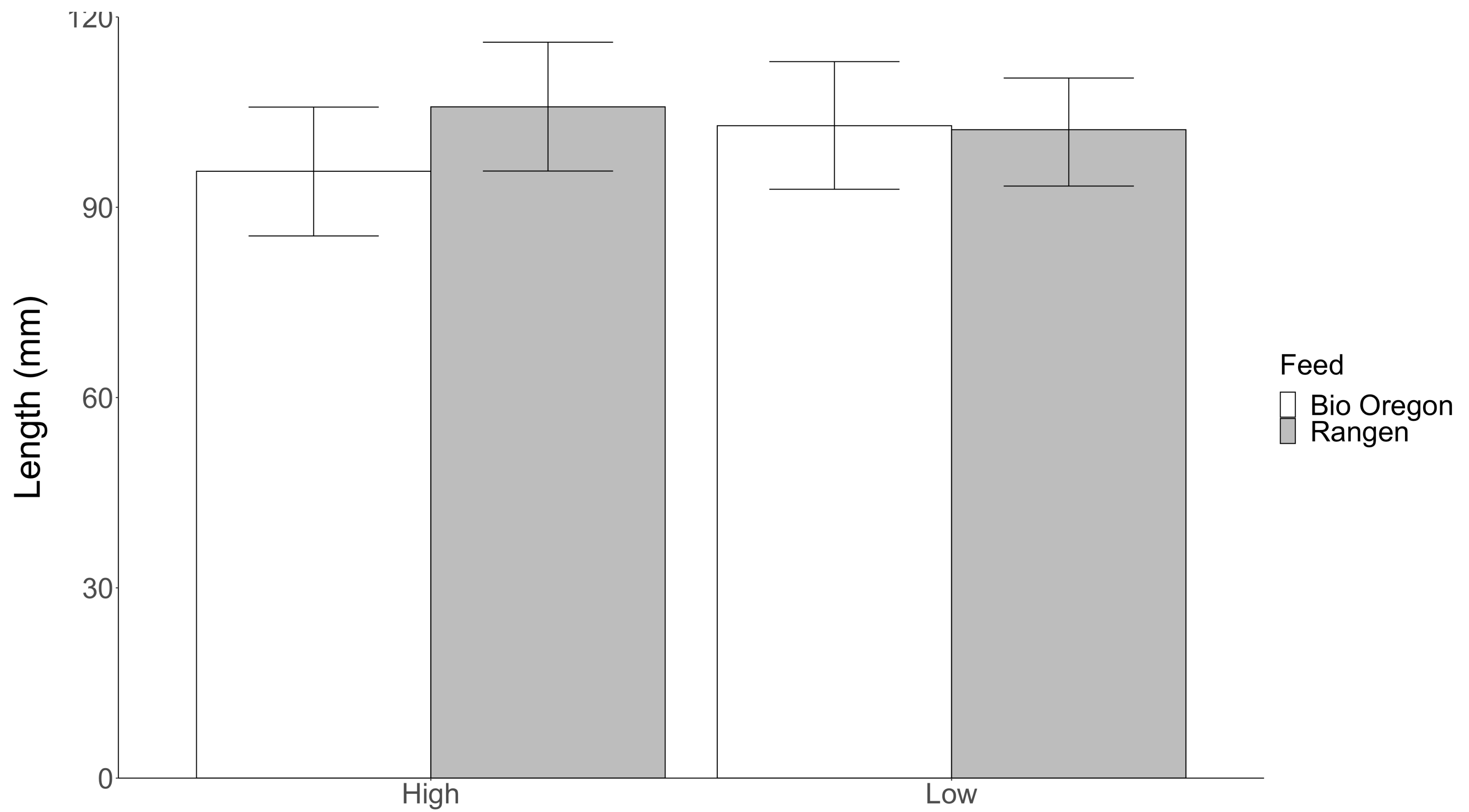

Figure 3.3. Mean fish length (mm; 95% credible interval bars) at the time of stocking for each rearing density and feed type.

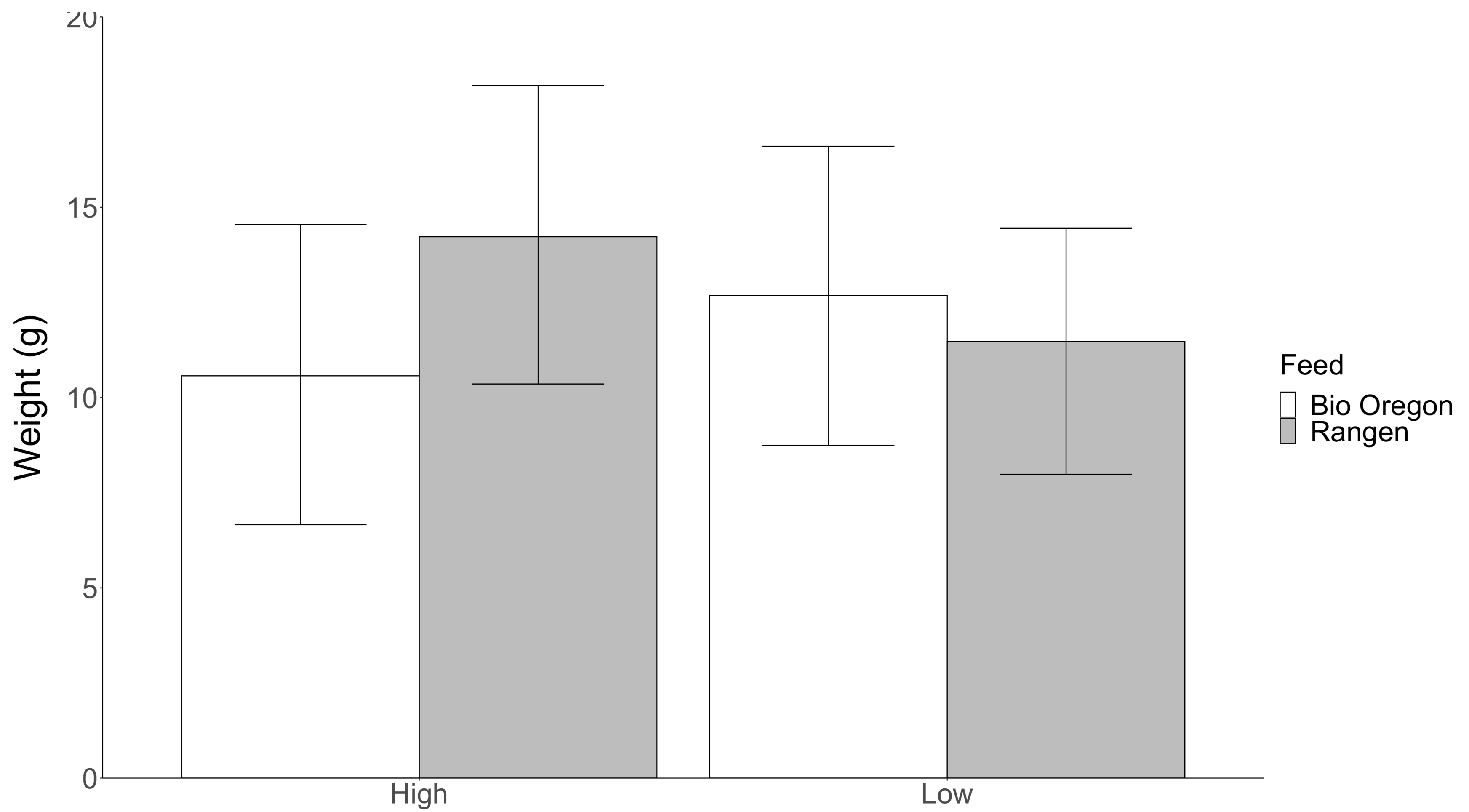

Figure 3.4. Mean fish weight (g; 95% credible interval bars) at the time of stocking for each rearing density and feed type.

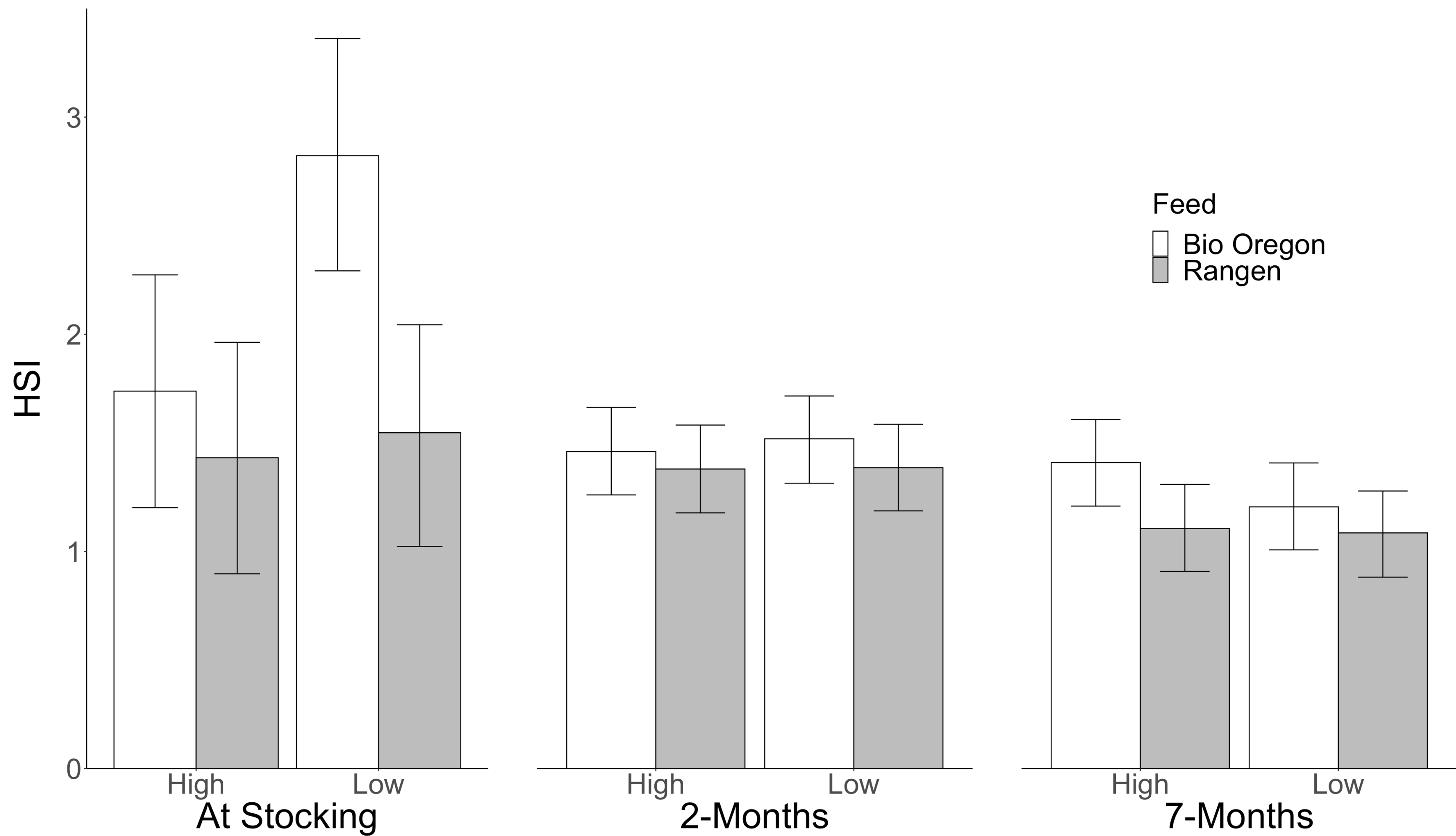

Figure 3.5. Mean hepatosomatic index (HSI; 95% credible interval bars) at the time of stocking, and two- and seven-months post-stocking for each rearing density and feed type.

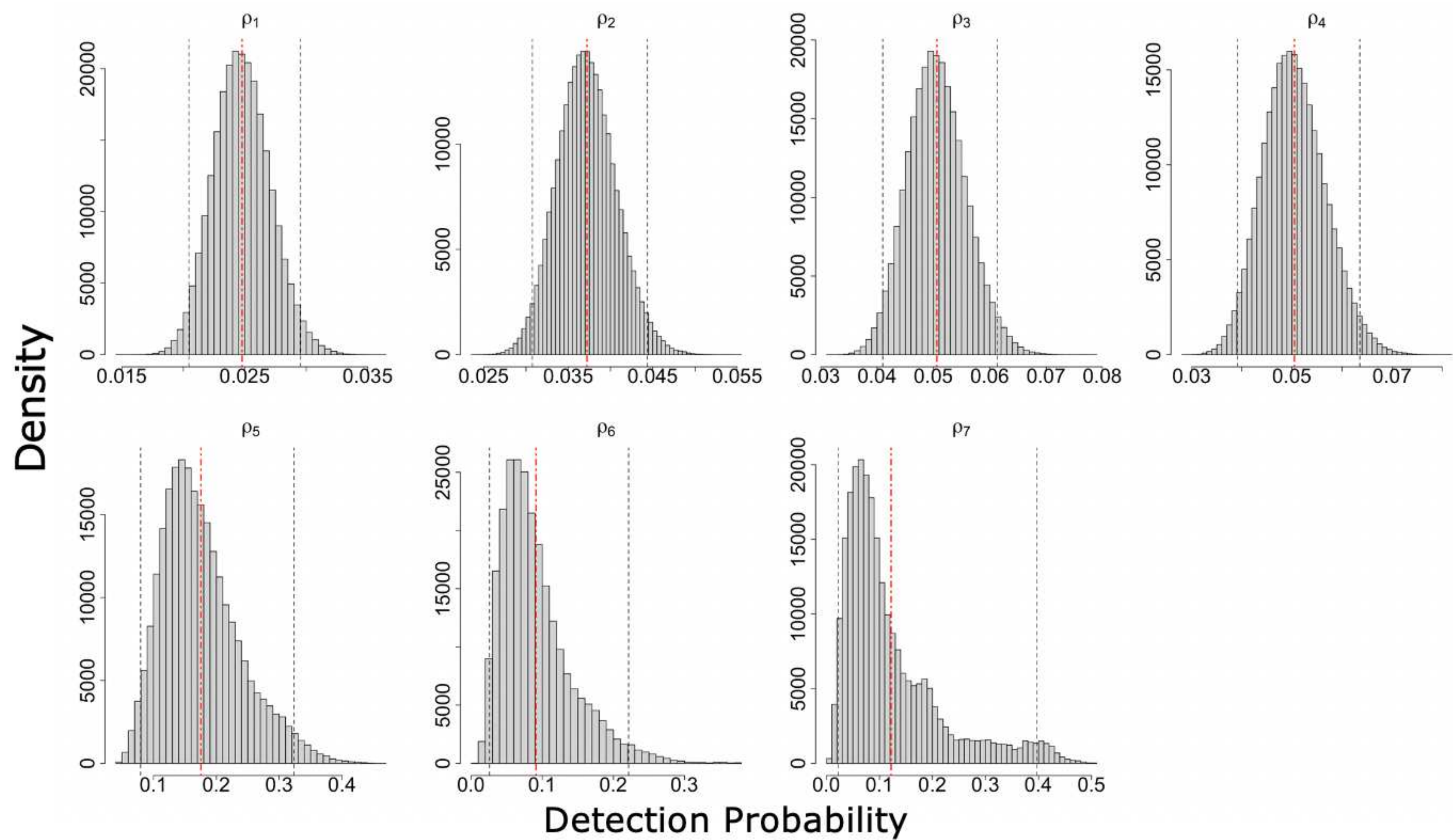

Figure 3.6. Posterior distributions for detection probability at each encounter time (ρ_t). Vertical red dashed lines denote the mean of the distribution and vertical dash black lines denote the upper and lower 95% credible interval bounds.

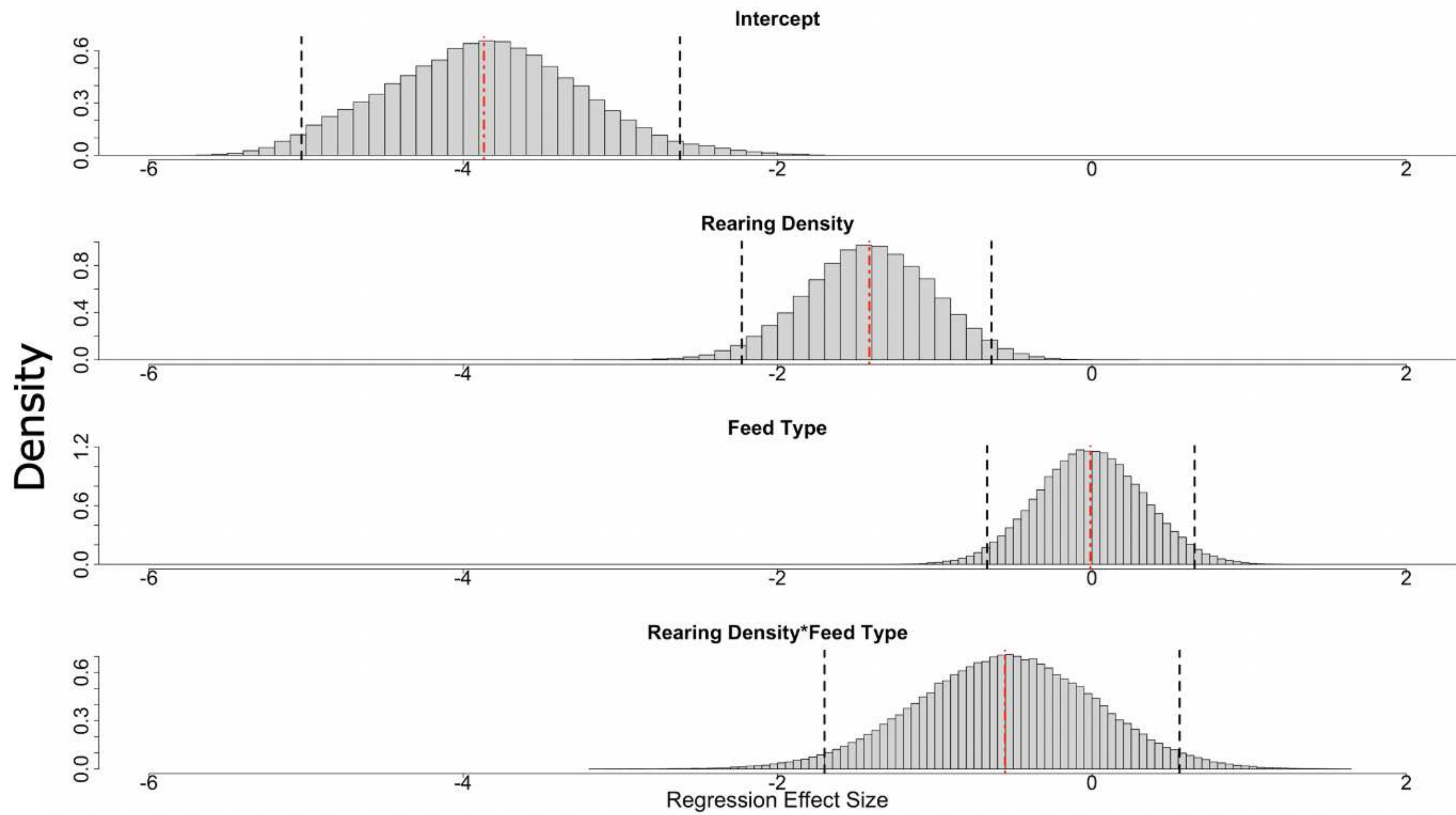

Figure 3.7. Posterior distribution for each factor (density, feed, density*feed) showing the effect on survival. Vertical red dashed lines denote the mean of the distribution and vertical dash black lines denote the upper and lower 95% credible interval bounds.

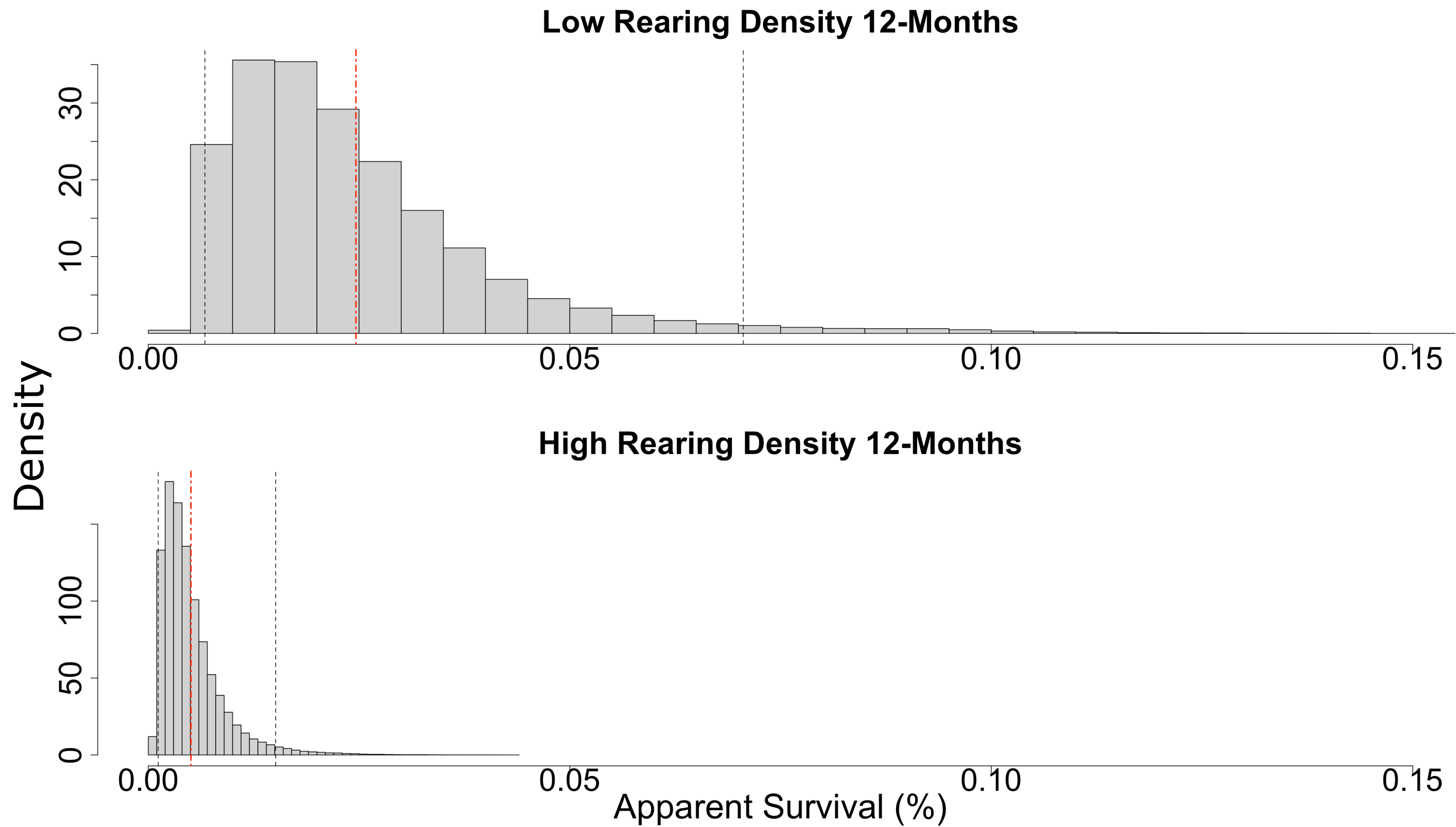

Figure 3.8. Posterior distributions for the 12-month interval survival (Φ) of each rearing density used to extrapolate two- and seven-month survival rates. Vertical red dashed lines denote the mean of the distribution and vertical dash black lines denote the upper and lower 95% credible interval bounds.

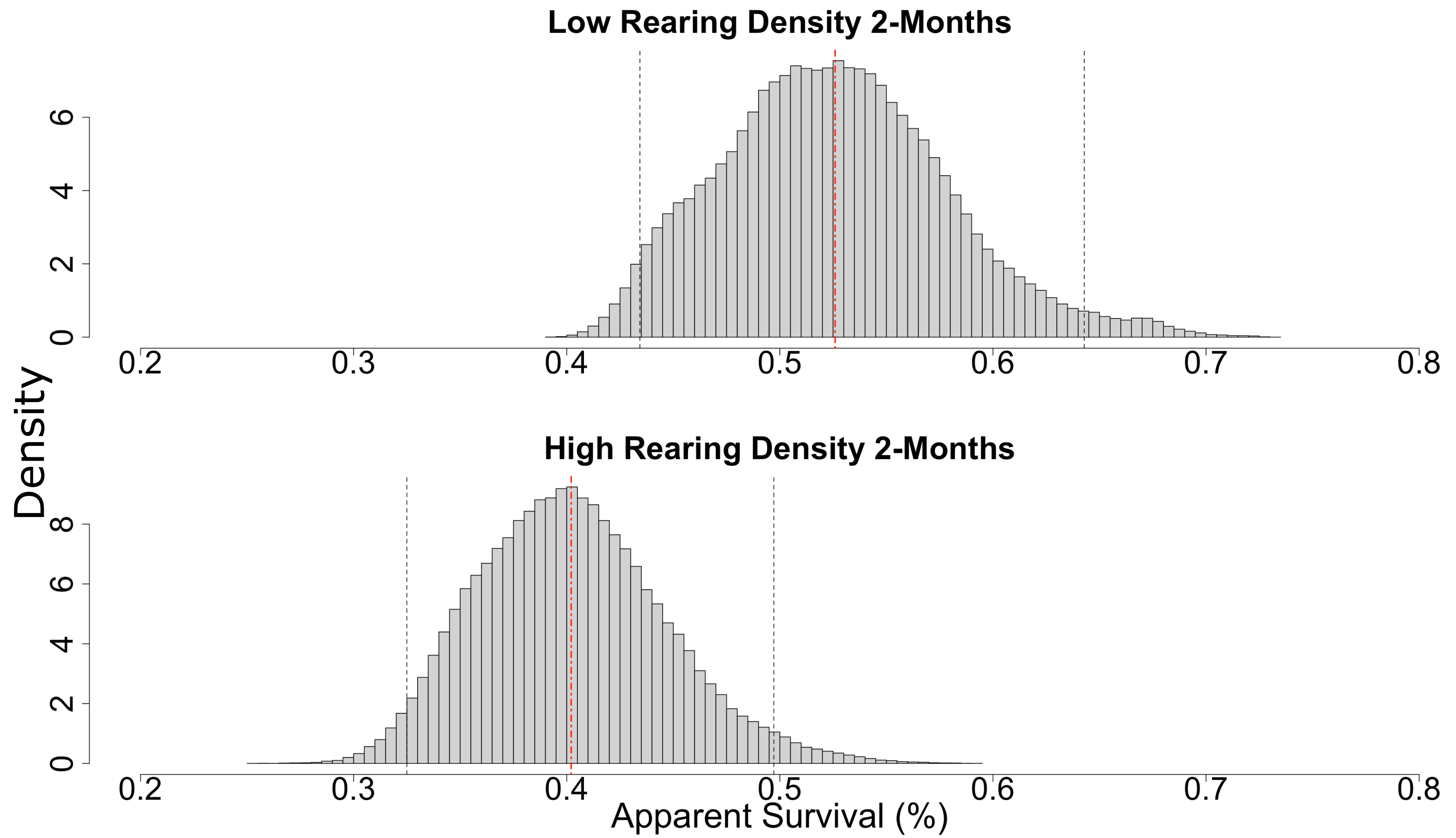

Figure 3.9. Posterior distributions for the two-month survival of each rearing density. Vertical red dashed lines denote the mean of the distribution and vertical dash black lines denote the upper and lower 95% credible interval bounds.

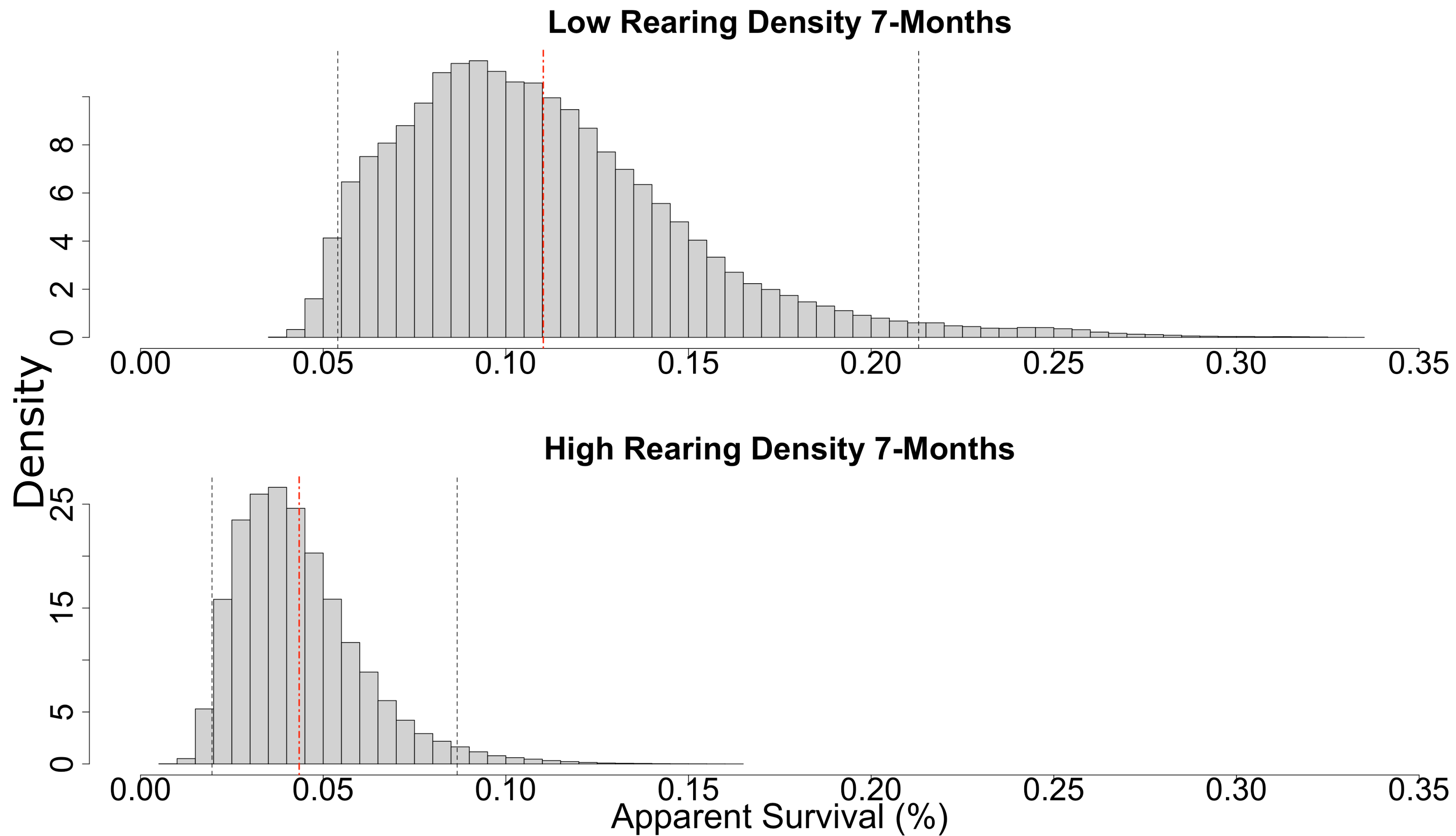

Figure 3.10. Posterior distributions for the seven-month survival of each rearing density. Vertical red dashed lines denote the mean of the distribution and vertical dash black lines denote the upper and lower 95% credible interval bounds.

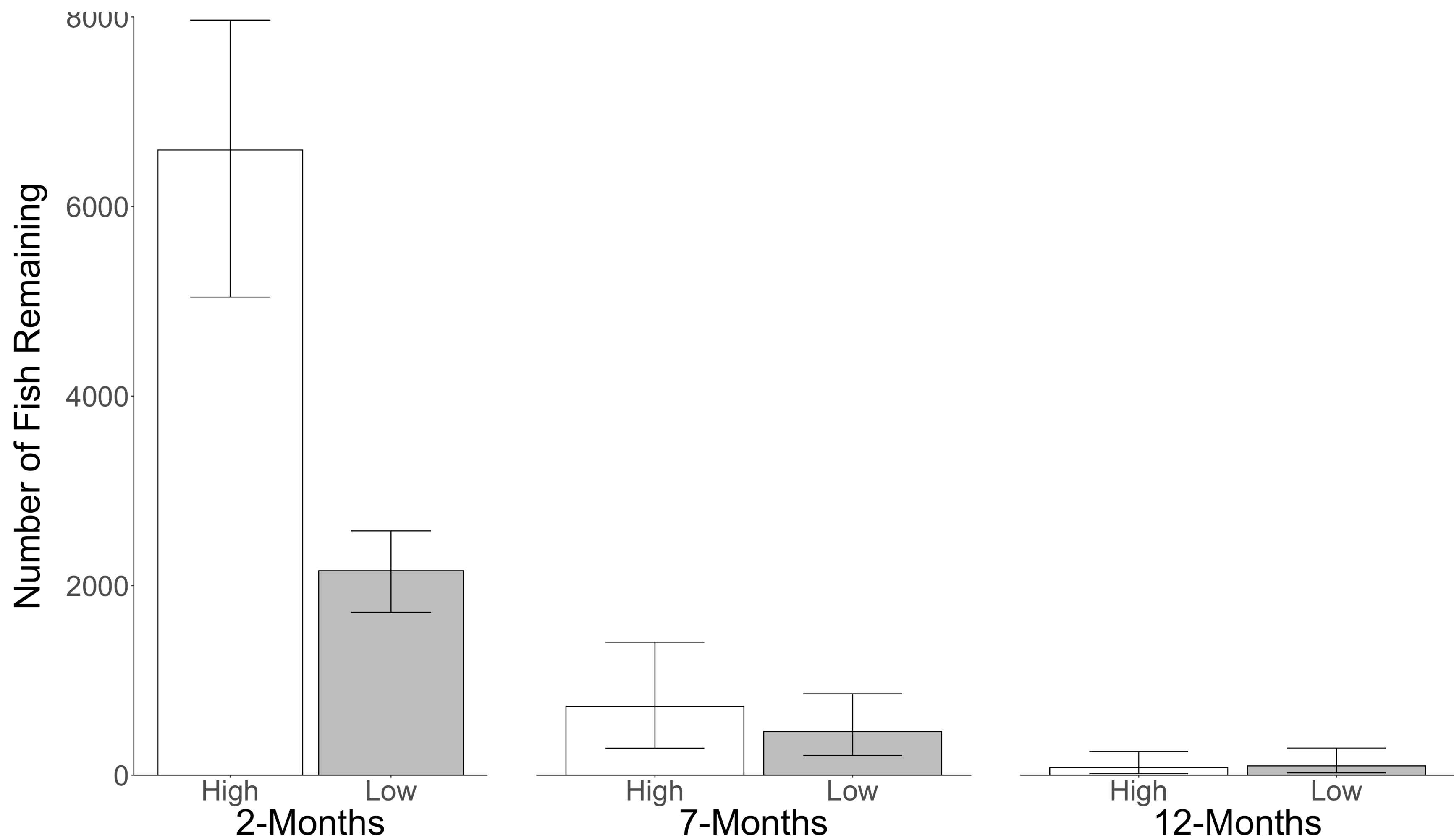

Figure 3.11. Estimated numbers of fish remaining (number of fish stocked times the estimated survival probability) from the high and low rearing density treatments (95% credible intervals) at two-, seven-, and twelve-months post stocking.

LITERATURE CITED

Alanärä, A., and E. Brännäs. 1996. Dominance in demand feeding behavior in Artic Charr and Rainbow Trout: the effect of stocking density. Journal of Fish Biology 48:242-254.

Anderson, R. O., and R. M. Neumann. 1996. Length, weight, and associated structural indices. Pages 447-483 *in* B. R. Murphy and D. W. Willis, editors. Fisheries techniques, 2nd edition. American Fisheries Society, Bethesda, Maryland.

Araki, H., B. Cooper, and M. S. Blouin. 2007. Genetic effects of captive breeding cause a rapid, cumulative fitness decline in the wild. Science 318:100-103.

Araki, H., B. A. Berejikian, M. J. Ford, and M. S. Blouin. 2008. Fitness of hatchery-reared salmonids in the wild. Evolutionary Applications 1(2):342-355.

Aprahamian, M. W., K. M. Smith, P. McGinnity, S. McKelvey, and J. Taylor. 2003. Restocking of salmonids – opportunities and limitations. Fisheries Research 62:211-227.

Atay, D., M. Olmez, A. S. Gulen, and S. Bekcan. 1988. The effects of different stocking rates on the growth of Rainbow Trout (*Salmo gairdneri* R.) in concrete canals at the Cifteler Sakaryabasi Production Station. Journal of Aquatic Production 2:223-233.

Barker, R. F., and G. B. Ayles. 1990. The effects of varying density and loading level on the growth of Artic Charr (*Salvalinus alpinus* L.) and Rainbow Trout (*Oncorhynchus mykiss*). World Aquaculture 21:58-62.

Barnes, M. E., and M. L. Brown. 2011. A Review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial coldwater disease prevention and treatment. The Open Fish Science Journal 4:1-9.

Barnes, M. E., M. M. Wipf, N. R. Domenici, W. M. Kummer, and R. P. Hanten. 2013. Decreased hatchery rearing density improves poststocking harvest and return to spawning of landlocked fall Chinook Salmon. North American Journal of Aquaculture 75:244-250.

Boydstun, L. B., and J. S. Hopelain. 1977. Cage rearing of Steelhead Rainbow Trout in a freshwater impoundment. Progressive Fish-Culturist 39:70-75.

Brockmark, S., B. Adriaenssens, and J. I. Johnsson. 2010. Less is more: density influences the development of behavioral life skills in trout. Proceedings of the Royal Society B 277:3035-3043.

Brockmark, S., and J. I. Johnsson. 2010. Reduced hatchery rearing density increases social dominance, postrelease growth, and survival in Brown Trout (*Salmo trutta*). Canadian Journal of Fisheries and Aquatic Sciences 67:288-295.

Brown, C., and K. Laland. 2001. Social learning and life skills training for hatchery reared fish. Journal of Fish Biology 59(3):471-493.

Brown, C., T. Davidson, and K. Laland. 2003. Environmental enrichment and prior experience of live prey improve foraging behavior in hatchery-reared Atlantic Salmon. Journal of Fish Biology 63(s1):187-196.

Charlesworth, D., and J. H. Willis. 2009. The genetics of inbreeding depression. Nature Reviews Genetics 10:783-796.

Christie, M. R., M. L. Marine, R. A. French, and M. S. Blouin. 2012. Genetic adaptation to captivity can occur in a single generation. Proceedings of the National Academy of Sciences 109(1):238-242.

Christie, M. R., M. L. Marine, S. E. Fox, R. A. French, and M. S. Blouin. 2016. A single generation of domestication heritably alters the expression of hundreds of genes. Nature Communications 7(10676)1-6.

Cipriano, R. C., and R. A. Holt. 2005. *Flavobacterium psychrophilum*, cause of bacterial cold-water disease and Rainbow Trout fry syndrome. United States Department of Interior, U.S. Geological Service, National Fish Health Research Laboratory. Fish Disease Leaflet No. 86.

Einum, S., and I. A. Fleming. 2001. Implications of stocking: Ecological interactions between wild and released salmonids. Nordic Journal of Freshwater Research 75:56-70.

Ellis, T., B. North, A. P. Scott, N. R. Bromage, M. Porter, and D. Gadd. 2002. The relationships between stocking density and welfare in farmed Rainbow Trout. Journal of Fish Biology 61:493-531.

Ewing, R. D., and S. K. Ewing. 1995. Review of the effects of rearing density on survival to adulthood for Pacific salmon. The Progressive Fish-Culturist 57:1-25.

Fagerlund, U. H. M., J. R. McBride, and E. T. Stone. 1981. Stress-related effects of hatchery rearing density on Coho Salmon. Transactions of the American Fisheries Society 110:644-649.

Fetherman, E. R., B. Neuschwanger, T. Davis, C. Praamsma, and D. Karr. 2019. Colorado hatchery feed experiments. Colorado Parks and Wildlife, Aquatic Wildlife Research Section. Fort Collins, Colorado.

FAO (Food and Agriculture Organization of the United Nations). 2020. The state of world fisheries and aquaculture. FAO, Rome.

Frankham, R. 2008. Genetic adaptation to captivity in species conservation programs. Molecular Ecology 17:325-333.

Gelman, A., and D. B. Rubin. 1992. Inference from iterative simulation using multiple sequences. Statistical Science 7:457-511.

Hansen, M. M., and K. L. Mensberg. 2009. Admixture analysis of stocked Brown Trout populations using mapped microsatellite DNA markers: Indigenous trout persist in introgressed populations. Biology Letters 5(5):656-659.

Heidelberger, P., and P. Welch. 1983. Simulation run length control in the presence of an initial transient. Operations Research 31:1109:1044.

Hobbs, N. T., and M. B. Hooten. 2015. Bayesian models a statistical primer for ecologists. Princeton University Press, Princeton, New Jersey.

Holm, J. C., T. Refstie, and S. Bo. 1990. The effect of fish density and feeding regimes on individual growth rate and mortality in Rainbow Trout (*Oncorhynchus mykiss*). Aquaculture 89:225-232.

Iguchi, K., K. Ogawa, M. Nagae, and F. Ito. 2003. The influence of rearing density on stress response and disease susceptibility of Ayu (*Plecoglossus altivelis*). Aquaculture 220:515-523.

Kavanagh, M., and D. E. Olson. 2014. The effects of rearing density on growth, fin erosion, survival, and migration behavior of hatchery winter Steelhead. North American Journal of Aquaculture 76:323-332.

Kelly-Quinn, M., and J. J. Bracken. 1989. Survival of stocked hatchery-reared Brown Trout, *Salmo trutta* L., fry in relation to the carrying capacity of a trout nursery stream. Aquaculture and Fisheries Management 20:211-226.

Kilambi, R. V., J. C. Adams, A. V. Brown, and W. A. Wickzer. 1977. Effect of stocking density and cage size on growth, feed conversion, and production of Rainbow Trout and Channel Catfish. Progressive Fish-Culturist 39:62-66.

Kindschi, G. A., C. E. Smith, and R. F. Koby Jr. 1991. Performance of two strains of Rainbow Trout reared at four densities with supplemental oxygen. Progressive Fish-Culturist 53:203-209.

Kincaid, H. L., W. R. Bridges, A. E. Thomas, and M. J. Donahoo. 1976. Rearing capacity of circular containers of different sizes for fry and fingerling Rainbow Trout. Progressive Fish-Culturist 38:11-17.

LaFrentz, B. R., and K. D. Cain. 2004. Bacterial Coldwater Disease. An Extension Bulletin for the Western Regional Aquaculture Center. University of Idaho, Moscow.

Leatherland, J. F., and C. Y. Cho. 1985. Effect of rearing density on thyroid and interrenal gland activity and plasma and hepatic metabolite levels in Rainbow Trout, *Salmo gairdneri* Richardson. Journal of Fish Biology 27:583-592.

Logan, S. H., and W. E. Johnston. 1992. Economics of commercial trout production. Aquaculture 100:25-46.

Lynch, M., and M. O'Hely. 2001. Captive breeding and the genetic fitness of natural populations. Conservation Genetics 2:363-378.

Makinen, T., and K. Ruohonen. 1990. The effect of rearing density on the growth of Finnish Rainbow Trout (*Oncorhynchus mykiss* Walbaum 1792). Journal of Applied Ichthyology 6:193-203.

Marchetti, M. P., and G. A. Nevitt. 2003. Effects of hatchery rearing on brain structures of Rainbow Trout, *Oncorhynchus mykiss*. Environmental Biology of Fishes 66:9-14.

Michel, C., Antonio, D. and R. P. Hedrick. 1999. Production of viable cultures of *Flavobacterium psychrophilum*: approach and control. Research in Microbiology 150:351-358.

Miller, S. A., E. J. Wagner, and T. Bosakowski. 1995. Performance and oxygen consumption of Rainbow Trout reared at two densities in raceways with oxygen supplementation. Progressive Fish-Culturist 57:206-212.

Nicola, S. J., and A. J. Cordone. 1973. Effects of fin removal on survival and growth of Rainbow Trout (*Salmo gairdneri*) in a natural environment. Transactions of the American Fisheries Society 102:753-758.

Nilsen, H., A. B. Olsen, Ø. Vaagnes, H. Hellberg, K. Bottolfsen, H. Skjelstad, and D. J. Colquhoun. 2011. Systemic *Flavobacterium psychrophilum* infection in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. Journal of Fish Diseases 34(5):403-408.

Olson, D. E., and M. Paiya. 2013. An evaluation of rearing densities to improve growth and survival of hatchery spring Chinook Salmon. Journal of Fish and Wildlife Management 4(1):114-123.

Pickering, A. D., T. G. Pottinger, J. P. Sumpter, J. F. Carragher, and P. Y. Le Bail. 1991. Effects of acute and chronic stress on the levels of circulating growth hormone in the Rainbow Trout, *Oncorhynchus mykiss*. General and Comparative Endocrinology 83:86-93.

Piper, R. G., I. B. McElwain, L. E. Orme, J. P. McCraren, L. G. Fowler, and J. R. Leonard. 1982. Fish Hatchery Management. United State Department of the Interior. Fish and Wildlife Service. Washington, D.C.

Procarione, L. S., R. P. Barry, and J. A. Malison. 1999. Effects of high rearing densities and loading rates on the growth and stress responses of juvenile Rainbow Trout. North American Journal of Aquaculture 61:91-96.

Pulkkinen, K., L. -R. Suomalainen, A. F. Read, D. Ebert, P. Rintamaki, and E. T. Valtonen. 2010. Intensive fish farming and evolution of pathogen virulence: the case of columnaris disease in Finland. Proceedings of the Royal Society B 277:593-600.

Purser, J., and P. Hart. 1991. Effect of stocking density on the growth of juvenile Rainbow Trout. Austasia Aquaculture 5:46-48.

Rosengren, M., E. Kvingedal, J. Naslund, J. I. Johnsson, and K. Sundell. 2017. Born to be wild: effects of rearing density and environmental enrichment on stress, welfare, and smolt migration in hatchery-reared Atlantic salmon. Canadian Journal of Fisheries and Aquatic Sciences 74:396-405.

Royle, J. A., and R. M. Dorazio. 2008. Hierarchical modeling and inference in ecology. Academic Press, London, UK.

Sirakov, I., and E. Ivancheva. 2008. Influence of stocking density on the growth performance of Rainbow Trout and Brown Trout grown in recirculation system. Bulgarian Journal of Agricultural Science 14(2):150-154.

Snyder, N. F. R., S. R. Derrickson, S. R. Beissinger, J. W. Wiley, T. B. Smith, W. D. Toone, and B. Miller. 1996. Limitations of captive breeding endangered species recovery. Conservation Biology 10(2):338-348.

Southwick Associates. 2017. The 2017 economic contributions of outdoor recreation in Colorado. Southwick Associates Fernandian Beach, Florida.

Starliper, C. E. 2011. Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. Journal of Advanced Research 2:97-108.

Suboski, M. D., and J. J. Templeton. 1989. Life skills training for hatchery fish: social learning and survival. Fisheries Research 7(4):343-352.

Unlu, A., and I. Baran. 1992. The effect of stocking density on the rates of length-weight up to the stage of 7-8 months in Rainbow Trout (*Oncorhynchus mykiss* W., 1792). Journal of Aquatic Production 6:81-94.

USDA (U. S. Department of Agriculture). 2020. Trout Production. USDA. ISSN: 1949-1948. Washington, D.C.

Wagner, E. J., T. Jeppsen, R. Arndt, M. D. Routledge, and Q. Bradwisch. 1997. Effects of rearing density upon Cutthroat Trout hematology, hatchery performance, fin erosion, and general health and condition. The Progressive Fish-Culturist 59:173-187.

Williamson, K. S., A. R. Murdoch, T. N. Pearsons, E. J. Ward, and M. J. Ford. 2010. Factors influencing the relative fitness of hatchery and wild spring Chinook Salmon (*Oncorhynchus tshawytscha*) in the Wenatchee River, Washington, USA. Canadian Journal of Fisheries and Aquatic Sciences 67:1840-1851.

Winfree, R. A., G. A. Kindschi, and H. T. Shaw. 1998. Elevated water, crowding, and food deprivation accelerate fin erosion in juvenile Steelhead. Progressive Fish-Culturist 60:192-199.

APPENDIX 1.1: META ANALYSIS EXPERIMENTAL INFECTION MODEL

Note: bold values indicate a vector

$i;\ observation: 1,\ 2,\ \cdots, 132$

$j;\ group: 1,\ 2,\ \cdots, 22$

$\mathrm{y}_{i,j}$: Number of fish dead

$p_{i,j}$: probability of mortality

$\boldsymbol{S}$: beta values from regression

$$\mathrm{y}_{i,j} \sim \mathrm{binomial}(N, p_{i,j})$$

$$p_{i,j} \sim \mathrm{beta}(\alpha_{i,j}, \beta_{i,j})$$

$$\alpha_{i,j} = \frac{\mu^2-\mu^3-\mu\sigma^2}{\sigma^2}$$

$$\beta_{i,j} = \frac{\mu-2\mu^2+\mu^3-\sigma^2+\mu\sigma^2}{\sigma^2}$$

$$\begin{gathered}\mu_{i,j} = \text{inverse logit}(S_{0,j} + S_1\mathrm{Dose}_{i,j} + S_2\mathrm{Weight}_i + S_3\mathrm{Hours}_{i,j} + S_4\mathrm{Subcutaneous}_{i,j} + \\ S_5\mathrm{Intraperitoneal}_{i,j} + S_6\mathrm{Intramuscular}_{i,j} + S_7\mathrm{Bath}_{i,j} + \\ S_8\text{CSF259-93}_{i,j} + S_9\mathrm{NCIMB1947}_{i,j} + S_{10}\mathrm{S21}_{i,j} + \\ S_{11}\mathrm{Dubois}_{i,j} + S_{12}\text{950106-1/1}_{i,j} + S_{13}\text{JIP 02-97}_{i,j} + \\ S_{14}\text{AVU-1T/07}_{i,j} + S_{15}\text{99/1A}_{i,j} + S_{16}\text{99/10A}_{i,j} + S_{17}\text{900406-1/3}_{i,j} + \\ S_{18}\text{FPG-101}_{i,j} + S_{19}\mathrm{None}_{i,j} + S_{20}\mathrm{Dose}_{i,j} * \mathrm{Weight}_{i,j} + \\ S_{21}\mathrm{Dose}_{i,j} * \mathrm{Subcutaneous}_{i,j} + S_{22}\mathrm{Dose}_{i,j} * \mathrm{Intraperitoneal}_{i,j} + \\ \mathrm{S}_{23}\mathrm{Dose}_{i,j} * \mathrm{Intramuscular}_{i,j} + \\ S_{24}\mathrm{Dose}_{i,j} * \mathrm{Bath}_{i,j})\end{gathered}$$

$$\boldsymbol{S}_0 \sim \mathrm{normal}(\mu_\xi, \sigma_\xi)$$

$$\mu_\xi \sim \mathrm{normal}(0, 1.96)$$

$$\sigma_\xi \sim \mathrm{normal}(0, 100)$$

$$\boldsymbol{S} \sim \mathrm{normal}(0, 1.96)$$

$$\sigma^2 \sim \mathrm{uniform}(0, 100)$$

Posterior

$$[\boldsymbol{p}, \boldsymbol{S}, \boldsymbol{S}_0, \sigma^2, \mu_\xi, \sigma_\xi \mid \boldsymbol{y}] \propto \prod_{i=1}^{n} \prod_{j=1}^{J} [y_{i,j} \mid p_{i,j}][p_{i,j} \mid \boldsymbol{S}, S_{0,j}, \sigma^2]\ [S_{0,j} \mid \mu_\xi, \sigma_\xi]\ [\boldsymbol{S}]\ [\sigma^2]\ [\mu_\xi]\ [\sigma_\xi]$$

APPENDIX 2.1: FLAVOBACTERIUM PSYCHRPHILUM PILOT STUDY

To conduct a dual resistance experiment using *Flavobacterium psychrophilum* and *Myxobolus cerebralis*, it was necessary to first estimate an appropriate dosage for *F. psychrophilum* exposure. I wanted a dose that would result in about a 50% mortality rate using susceptible Rainbow Trout that were both able to contract bacterial coldwater disease (BCWD) and naïve, i.e., never previously exposed to *F. psychrophilum*.

METHODS

Culture and Harvesting of Flavobacterium psychrophilum

The CSF259-93 isolate of *F. psychrophilum* was obtained from Dr. Kenneth Cain's laboratory located at the University of Idaho. The bacterium was initially cultured at the Colorado Parks and Wildlife (CPW) Aquatic Animal Health Lab (Brush, Colorado) on tryptone yeast extract salt (TYES; 0.4% tryptone, 0.04% yeast extract, 0.02% calcium chloride, 0.05% magnesium sulfate; pH between 7.1 and 7.3) agar plates. Once a pure colony was isolated, 100 ml of modified TYES (including 0.2% dextrose) broth was inoculated with *F. psychrophilum* in a baffled flask and placed on a shaker table. Growth of bacteria was maintained by passing bacteria by inoculation of additional 100-mL flasks containing modified TYES continued every 24 – 36 hours to maintain a culture at log phase growth (maximum population growth rate). Optical density (OD) measurements, which are an index of the number of bacteria in the media at a point in time, were taken at a wavelength of 595 nm starting at 22 hours post inoculation (PI) through 32 hours PI. Ten-fold serial dilutions, which are made by taking 9-parts of TYES and adding 1-part bacteria in media at a higher concentration to create a ten-fold reduction of the bacteria, were used to conduct plate counts (the way to estimate bacteria concentration) every two hours, starting at 22 hours PI through 32 hours PI to confirm the population growth trajectory of the bacteria. The goal was to harvest bacteria at their maximum population growth rate. Maximum population growth rate can be estimated by determining which OD value is in the middle of the middle of the growth curve. As culture time increases, resources in the culture media decrease, leading to a

combination of both actively replicating bacteria and inactive bacteria that are in a stationary growth phase (Wang et al. 2015). Bacteria in the stationary growth phase may have decreased virulence, and *F. psychrophilum* harvested at 18 and 24 hours has been shown to cause higher mortality when exposed to fish than bacteria harvested at 48 hours (Aoki et al. 2005). An additional OD measurement was taken at 45 hours PI to determine if bacteria had reached a stationary phase with little to no population growth, indicating they were no longer replicating.

To produce a large volume of bacteria, I inoculated two 1,500-mL baffled flasks containing modified TYES with bacteria from the cultured-broth flasks. Bacteria (0.34 OD, 26-hours) were harvested by placing 40 mL of broth culture from a 1,500-mL flask into each of six 50-ml centrifuge tubes. The tubes were then placed in a centrifuge and spun at 8,000 g (8,873 relative centrifugal forces) for 10 minutes. The remaining liquid was decanted into a discard flask, leaving a concentrated pellet of *F. psychrophilum* bacteria. An additional 40 mL of culture broth from the same 1,500-mL flask was then placed back into the same 50-mL centrifuge tubes, and the process was repeated. The culture in both 1,500-mL flasks was concentrated into bacteria pellets using this process, resulting in 12 50-mL centrifuge tubes with concentrated bacteria pellets.

Each bacteria pellet was re-suspended, using a pipette to gently mix the bacteria pellet with 10 mL TYES into separate 15 mL vials. All concentrated and re-suspended TYES media with re-suspended pellets were then pooled together into a 250 mL vial, and 50 mL of modified TYES and 10% glycol was added (Michel and Garcia 2003). To allow for easy dosage manipulation, potential future bacteria propagation, and to reduce freeze-thaw events, the bacteria was placed into 31 15-mL vials containing 5 mL of bacteria. Number of colony forming units (CFU/mL) was determined by plating 10-fold serial dilutions on TYES agar plates prior to freezing. All vials were kept in a -80°C freezer.

Exposure Challenges

German x Harrison Lake Rainbow Trout were exposed to F. psychrophilum in two challenge experiments conducted in the Colorado Cooperative Fish and Wildlife Research Unit Quonset hut wet lab (Fort Collins, Colorado). The first challenge experiment consisted of five treatments including low,

medium, and high bacterial dosages, a mock TYES injection, and a control (no injection), and were replicated in triplicate (15 experimental tanks). Exposure dosages were prepared by thawing preserved bacteria and using serial dilutions. Bacterial dosages in the first experiment consisted of 5×106 CFU/mL (low), 5×107 CFU/mL (medium), and 5×108 CFU/mL (high). Dosages used in the subsequent experiment were adaptively determined based on results from the first experiment. For example, if the high dose in the first experiment resulted in 90-100% mortality, we reduced the maximum dosage, and proportionally lowered the low and medium dosages, in the subsequent experiment (Table A2.1.1). Twenty-five fish were held in each tank to allow assessment of mortality, and to standardize and compare to other published studies.

To start an experiment, fish were moved from the CPW Bellvue Fish Research Hatchery (CPW BFRH) to the Quonset hut wet lab. Temperature ranged between 12.3° and 13.4° C in the experimental tanks at the Quonset hut wet lab. After a two-day acclimation period, Rainbow Trout in the bacterial challenge tanks were injected subcutaneously at the dorsal midline posterior to the dorsal fin with virulent F. psychrophilum (CSF259-93; 25 µL). Rainbow Trout in mock injection tanks were subcutaneously injected at the dorsal midline posterior to the dorsal fin with 25 µL of TYES broth media to ensure that exposure to the bacteria and not the physical damage from injection caused observed mortality. No injections occurred for fish in the control tanks. At the time of injection, fish were weighed. Fish were monitored at least twice a day, moribund and dead fish were removed, and lengths and weights were recorded daily for 28 days post-exposure. Raw mortality data was transformed into cumulative percent mortality (CPM) curves to examine trends in mortality over time.

Statistical Analysis

Dr. John Drennan's data (Table A2.1.1) was combined with my data before any analysis was conducted. Dr. Drennan conducted his experiments using the same methods as stated above. His data allowed me to increase my sample size and helped with inference in the dose*weight effect. The analyzed data is for all five experiments combined.

Prior to statistical modeling of mortality, all covariates (bacterial dosage, weight of fish prior to exposure and the interaction between dose and weight [dose*weight]) were examined for correlation using the Pearson correlation method. The large differences in units and scale between weight and dose prompted standardizing all covariate information prior to analysis by subtracting the mean of the covariate group and dividing by the standard deviations of covariate group (Table A2.1.2). Standardization of each covariate occurred by combining all data and then subtracting the mean of the covariate group and dividing by the standard deviation of the covariate group. Mortality was analyzed by using a Bayesian binomial beta regression model (Appendix 2.2) using Just Another Gibbs Sampler (JAGS) within program R (version 4.0.3). The response data consisted of the total number of fish dead in each tank. The predictor variables were bacterial dose, fish weight prior to exposure, and dose*weight. The Bayesian model allows for the use of available prior information and estimation of the probability of regression coefficient parameters being positive or negative. The binomial beta regression allows for added variation not accounted for with a traditional binomial regression model. Vague prior information was used for the standard deviation parameter (σ) with a mean of zero and a variance of 0.34. Posterior inference for model parameters and derived quantities were based on two chains of 500,000 Markov chain Monte Carlo (MCMC) samples following convergence after a burn in period of 20% of total iterations. Convergence was determined by visual inspection of mixed trace plots for model parameters associated with the intercept, dose, weight, dose*weight, any odds of mortality and probability of mortality, and the use of Gelman and Rubin diagnostic (Gelman and Rubin 1992). Gelman and Rubin diagnostic values less than 1.1 indicate model convergence (Hobbs and Hooten 2015). Inferences about morality were evaluated by examining the posterior distribution for each covariate allowing for a calculated mean and 95% credible interval for each factor. Posterior predictive checks also known as Bayesian p-values were used, looking at the mean of the data (mortality) and standard deviation of the data, to determine lack of fit. The posterior predictive check tests if the distribution of the data created by the model is more extreme than the distribution of the observed data. Bayesian p-values less than 0.1 and greater than 0.9

indicate lack of fit (Hobbs and Hooten 2015). I present the multiplicative change in odds of mortality (eregression coefficient), mean effect size and the associated 95% credible intervals for each covariate.

RESULTS

Culture and Harvesting of Flavobacterium psychrophilum

Maximum bacterial replication was estimated to be obtained at an OD of 0.3 (Figure A2.1.1). Harvest and bacterial concentration for injection occurred at an OD of 0.34 corresponding to a culture time of 26 hours. Final concentration of the bacteria was 8.1×10^9 CFU/mL.

Exposure and mortalities

Prior to exposure, fish weights across all five experiments ranged between 5.44g and 15.66g (Table A2.1.1). After the subcutaneous injections with *F. psychrophilum*, it was typical to see a range of bruising, from none to mild discoloration, around the injection site. If bruising was present, it either disappeared in one to two days or developed into a red welt at the injection site. Red welts would then get inflamed, or boils would form. Boils at the injection site would typically increase in size until they ruptured, resulting in the classical bacterial coldwater disease lesions with yellow bacteria around the edges. There was some bruising in the mock-injection treatments but no red welts, boils or lesions. Mortality typically started between five- and ten-days post-exposure to *F. psychrophilum*, though as late as 15 days post-exposure for some doses, and generally concluded 7-14 days after it began (Figure A2.1.2 and Figure A2.1.3). Mock injections did not result in increased mortality, suggesting that exposure to *F. psychrophilum* was the cause of the observed patterns in mortality in the challenge experiments (Figure A2.1.3).

Statistical Analysis

Visual inspection of trace plots and the result that all Gelman and Rubin diagnostic values were equal to 1.0 for all model parameters indicated that the two MCMC chains mixed and converged to the target distributions. The Bayesian p-value for the mean of the data was (0.52) and Bayesian p-value for the standard deviation of the data was (0.43) indicating proper model fit.

The posterior distributions indicated that both dose and weight have a positive effect on mortality (> 98% of the coefficient distribution was positive; Figure A2.1.4), and that dose*weight has a negative effect on mortality (> 78% of the coefficient distribution was negative; Figure A2.1.4). The mean value for the regression coefficients calculated for the intercept, dose, weight, and dose*weight ranged between -0.33 and 0.97 (Table A2.1.3). It appears that mortality is dose and weight dependent, with a decrease in odds of mortality of 0.72 for the dose*weight interaction (Table A2.1.4). For any dose, mortality decreased as weight increased. The mean of the posterior distribution for the standard deviation associated with mean mortality is 0.25 (0.21, 0.30; 95% credible interval). Due to the large variability in mortality, predicting accurate mortality is difficult. The probability of mortality ranged between 23.57% and 98.59% based on the dosage and weight of the fish at the time of exposure (Table A2.1.5). A mortality that would be a good starting point for future experiments, such as a dual exposure experiment, is 50% mortality. A mortality of 50% for susceptible fish should allow for differences to be determined between susceptible and resistant fish. The doses that have 95% credible intervals that cover 50% probability of mortality for the range of weights used within the experiment are: $1.5x10^7$ (6.96 g), $1.50x10^8$ (7.43 g), $5.00x10^7$ (8.43 g), $5.00x10^6$ (10.47 g), and $1.14x10^4$ (13.83 g) CFU/mL (Table A2.1.5).

The cultured and prepared frozen *F. psychrophilum* samples from this experiment were saved for use in other *F. psychrophilum* exposure experiments following Michel and Garcia (2003), such as the dual exposure and F1-generation cross evaluation experiments in this chapter. The known CFU/mL of the frozen stock allows for different *F. psychrophilum* doses to be created. Analysis of the data indicate that mortality is dose dependent and fish weight affects mortality when exposed to *F. psychrophilum*. Weight was therefore taken into account when setting dosage for the dual exposure experiment. The large variability associated with mortality leads to difficulty when predicting mortality for given doses. However, estimated mortality for the doses used within the experiments suggests doses between $1.14x10^4$ and $1.5x10^8$ will result in 50% mortality for strains susceptible to *F. psychrophilum* infection, and therefore the dosage used in the dual exposure experiment fell within this range.

Table A2.1.1. *Flavobacterium psychrophilum* exposure doses (CFU/mL; replicates in parentheses) and fish weights (grams or grams ± SD) associated with the challenge experiments conducted by Dr. John Drennan and Brian Avila.

Experiment	Dose	Fish Weight (g)	Conducted by:
	0 (Mock; 1)	6.12	
1	5.00 × 106 (3)	6.81	Drennan
	5.00 × 107 (3)	6.96	
	5.00 × 108 (3)	6.44	
	0 (Mock; 1)	7.64	
2	5.00 × 107 (3)	8.39	Drennan
	1.50 × 108 (3)	9.71	
	0 (Mock; 1)	5.44	
3	5.00 × 107 (3)	7.45	Drennan
	1.50 × 108 (3)	7.43	
	0 (Control; 1)		
	0 (Mock; 2)		
	5.00 × 105 (3)		
4	5.00 × 106 (3)	10.45 ± 0.52	Avila
	5.00 × 107 (3)		
	5.00 × 108 (3)		
	0 (Mock; 1)		
5	6.85 × 103 (3)		
	1.40 × 104 (3)		
	4.80 × 105 (1)	13.96 ± 0.78	Avila
	1.10 × 106 (3)		
	1.19 × 107 (3)		
	1.90 × 108 (1)		

Table A2.1.2. Values used to standardize each covariate data group by subtracting the mean of the covariate group and dividing by the standard deviation of the covariate group. Standardization provided a unitless value for each covariate that could be used for comparisons across covariates in the analysis.

Covariate Data	Mean	Standard Deviation
Dose	89830991.5	156524161
Weight	10.0877358	2.89289009
Dose*Weight	751646334	1367948917

Table A2.1.3. Posterior regression coefficients (S) from standardized data with the lower and upper 95% credible interval bounds.

Parameter	Mean	Lower	Upper
$S_{Intercept}$	0.45	0.12	0.78
S_{Dose}	0.97	0.089	1.88
S_{Weight}	0.74	0.37	1.10
$S_{Dose*Weight}$	-0.33	-1.17	0.43

Table A2.1.4. The multiplicative change of the odds associated with each regression parameter.

Parameter	Odds
Intercept	1.57
Dose	2.64
Weight	2.094
Dose*Weight	0.72

Table A2.1.5. Average probability of mortality with the associated 95% credible intervals (CIs) for *F. psychrophilum* doses ranging between 0 and 5.0×10^8 CFU/mL and weights ranging between 5.44 g and 15.66 g organized by weight (low to high). Bolded rows denote doses and weights that have 95% credible intervals that include the goal 50% probability of mortality.

Exp.	Dose	Weight	SD (Wt.)	Prob. of Mort.	Lower CI	Upper CI
3	0	5.44	0	0.035	0.0013	0.15
1	0	6.12	0	0.32	0.16	0.50
1	1.50×10^8	6.44	0	0.93	0.86	0.97
1	1.50×10^6	6.81	0	0.24	0.15	0.33
1	**1.50×10^7**	**6.96**	**0**	**0.57**	**0.46**	**0.67**
3	**1.50×10^8**	**7.43**	**0**	**0.56**	**0.45**	**0.66**
3	5.00×10^7	7.45	0	0.33	0.24	0.42
2	0	7.64	0	0.072	0.0083	0.193
2	**5.00×10^7**	**8.39**	**0**	**0.40**	**0.30**	**0.51**
2	1.50×10^8	9.71	0	0.67	0.57	0.77
4	5.00×10^8	10	0.6007	0.99	0.95	1
4	5.00×10^5	10.4	0.5696	0.67	0.56	0.76
4	0	10.46	0	0.075	0.019	0.16
4	**5.00×10^6**	**10.47**	**0.1253**	**0.62**	**0.52**	**0.72**
4	5.00×10^7	10.94	0.3602	0.92	0.85	0.97
5	6.85x103	12.97	0.4272	0.27	0.18	0.37
5	**1.14×10^4**	**13.83**	**0.5565**	**0.43**	**0.34**	**0.53**
5	0	13.96	0	0.055	0.0033	0.17
5	1.19×10^7	14.09	0.1185	0.98	0.93	1
5	1.10×10^6	14.22	0.7174	0.86	0.76	0.92
5	1.90×10^8	14.58	0	0.99	0.93	1
5	4.80×10^5	15.66	0	0.84	0.68	0.95

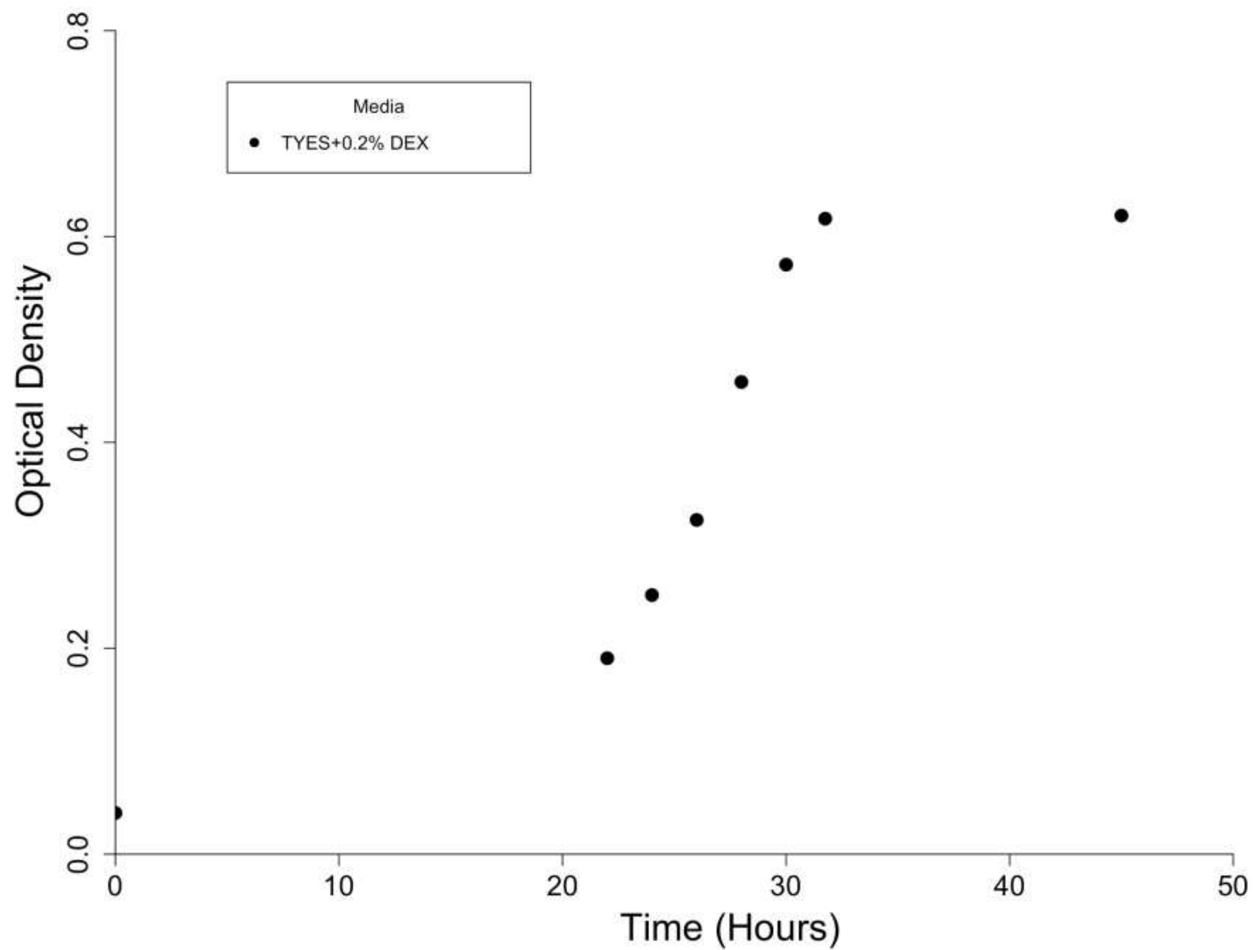

Figure A2.1.1. Growth curve for *Flavobacterium psychrophilum* (CSF 259-93) cultured in TYES + 0.2% DEX media, produced using optical density measurements taken every two hours starting at 22 hours post-inoculation as an index of bacterial growth over time.

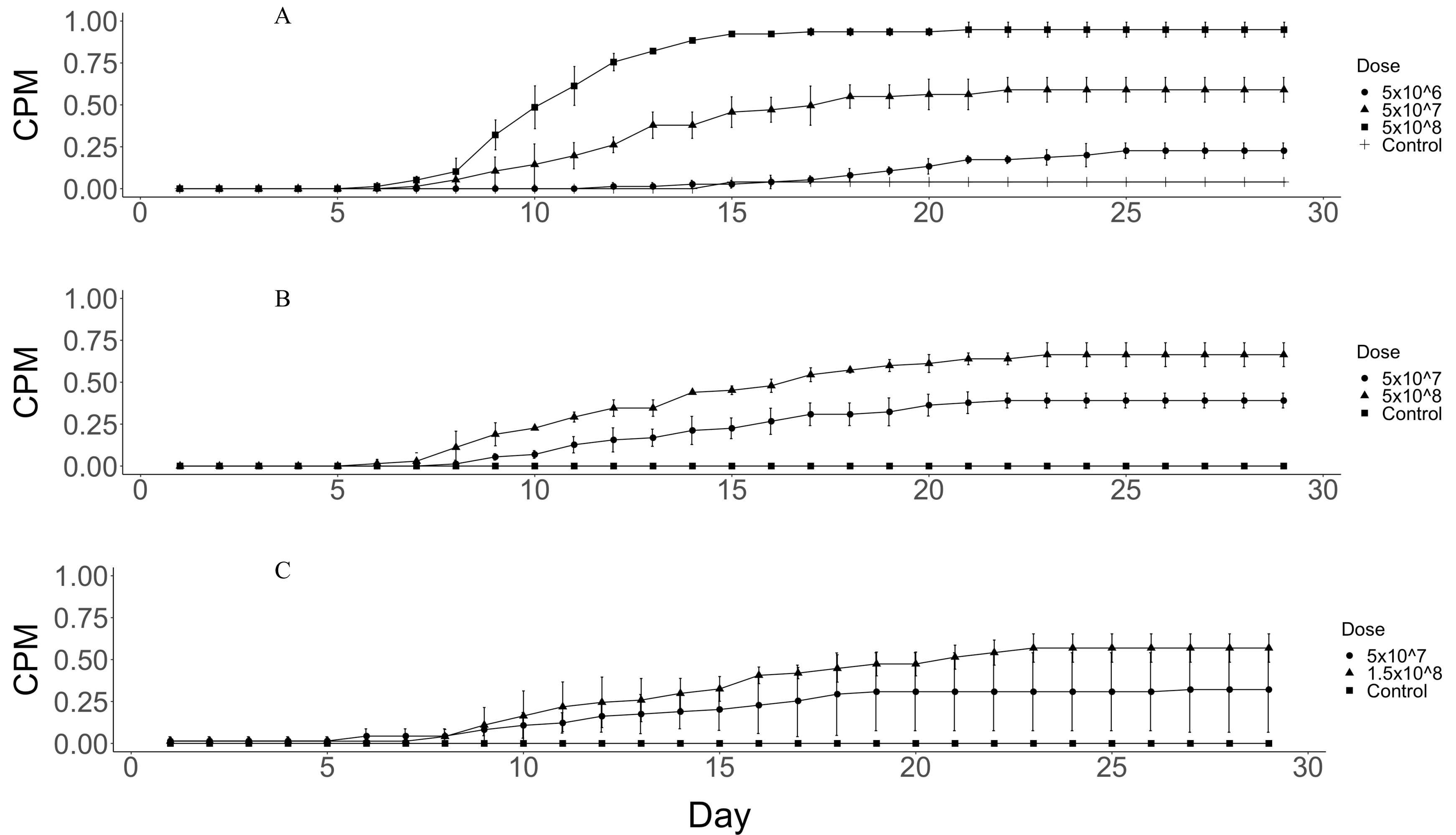

Figure A2.1.2. Cumulative percent mortality (CPM; standard deviation bars) over 28 days constructed using data from Dr. John Drennan's challenge experiments one (A), two (B) and three (C).

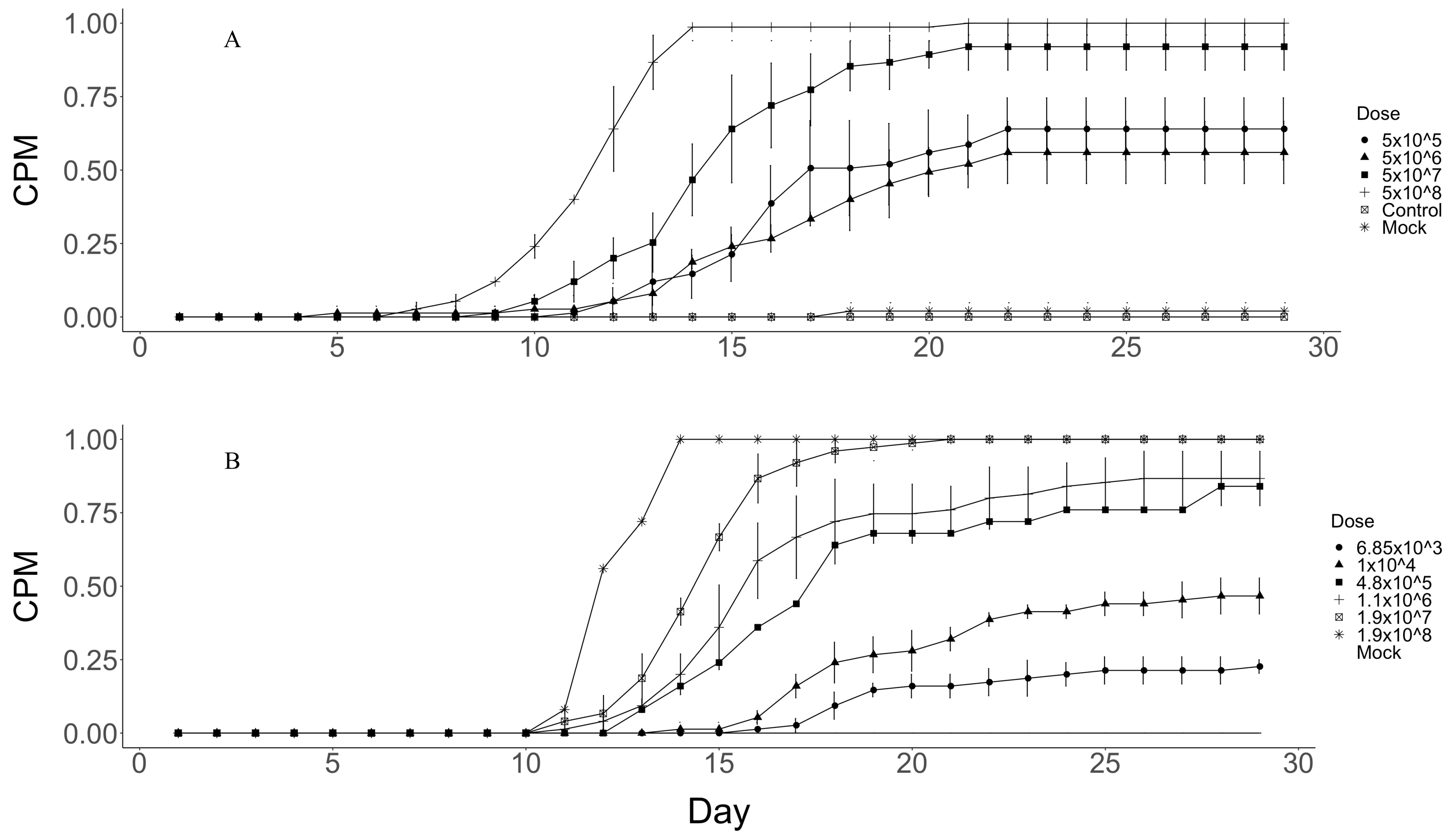

Figure A.2.1.3. Cumulative percent mortality (CPM; standard deviation bars) over 28 days constructed using data from Brian Avila's challenge experiments four (A) and five (B).

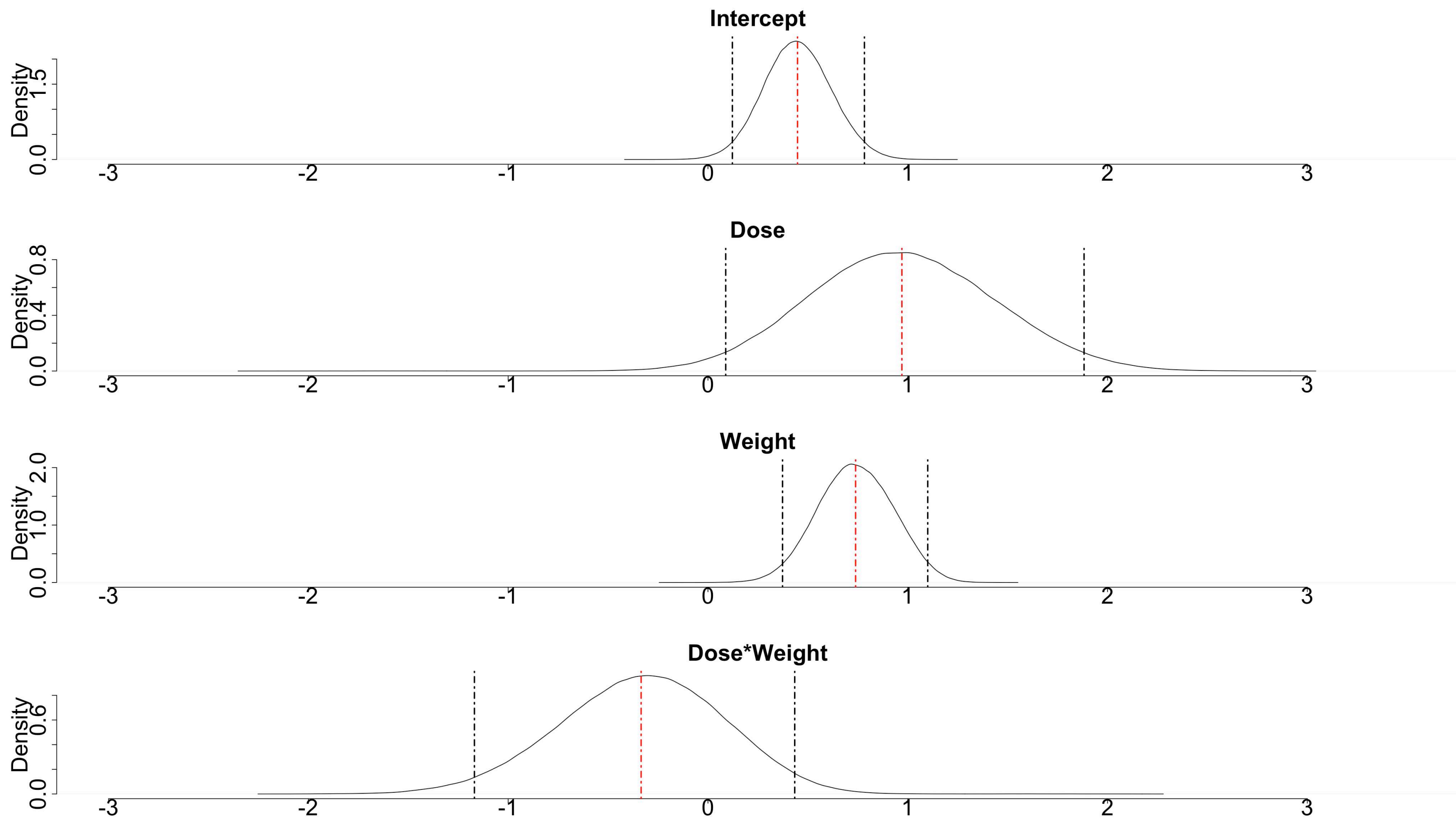

Figure A2.1.4. Posterior distributions for each of the regression coefficients (Intercept, Dose, Weight, and Dose*Weight). Red dotted vertical line shows the mean of the distribution and the black dotted lines denote the upper and lower 95% credible intervals.

LITERATURE CITED

Aoki, M., M. Kondo, K. Kawai, and S. Oshima. 2005. Experimental bath infection with *Flavobacterium psychrophilum*, inducing typical signs of Rainbow Trout *Oncorhynchus mykiss* fry syndrome. Diseases of Aquatic Organisms 67(1-2):73-79.

Gelman, A., and D. B. Rubin. 1992. Inference from iterative simulation using multiple sequences. Statistical Science 7(4) 457-472.

Michel, C., and C. Garica. 2003. Virulence stability in *Flavobacterium psychrophilum* after storage and preservation according to different procedures. Veterinary Research 34:127-132.

Hobbs, N. T., and M. B. Hooten. 2015. Bayesian models: a statistical primer for ecologists. Princeton University Press. Princeton, New Jersey.

Wang, L., D. Fan, W. Chen, and E. M. Terentjev. 2015. Bacterial growth, detachment and cell size control on polyethylene terephthalate surfaces. Scientific Reports 5(1):1-11.

APPENDIX 2.2: EXPERIMENTAL INFECTION MODEL

Note: bold values indicate a vector

y_i : Number of fish dead

p_i : Probability of mortality

N_i : Number of fish in each tank

$\boldsymbol{S}$: Regression coefficients

$\boldsymbol{\mu}$: Mean probability of mortality

$$\mathrm{y}_i \sim \text{binomial}(N_i, p_i)$$

$$p_i \sim \text{beta}(\alpha_i, \beta_i)$$

$$\alpha_i = \frac{\mu^2 - \mu^3 - \mu\sigma^2}{\sigma^2}$$

$$\beta_i = \frac{\mu - 2\mu^2 + \mu^3 - \sigma^2 + \mu\sigma^2}{\sigma^2}$$

$$\mu_i = \text{inverse logit}(S_0 + S_1\text{Dose}_i + S_2\text{Weight}_i + S_3(\text{Dose}_i * \text{Weight}_i))$$

$$S_1 \sim \text{normal}(0, 1.96)$$

$$S_{\ 2} \sim \text{normal}(0, 1.96)$$

$$S_3 \sim \text{normal}(0, 1.96)$$

$$S_4 \sim \text{normal}(0, 1.96)$$

$$\sigma \sim \text{uniform}(0, 0.33)$$

Posterior

$$[\ \boldsymbol{S}, \sigma^2, \boldsymbol{p} \mid \boldsymbol{y}] \propto \prod_{i=1}^{n} [y_i \mid p_i][p_i \mid S_i, \sigma^2]\ [\boldsymbol{S}]\ [\sigma^2]$$

APPENDIX 3.1: FEED INFORMATION

Table A3.1.1. Feed size and corresponding percent (%) crude protein, % crude fat, and description of the feed from the catalogs provided by Bio Oregon and Rangen (from Fetherman et al. 2019).

Feed Size	% Crude Protein	% Crude Fat	Description
		BIO OREGON	
#0 #1 and #2	53 52	18 20	Combines traditional dietary values with an increased level of alternative ingredients to reduce cost; contains an enhanced vitamin pack and natural pigment to promote healthy fish and natural coloration; natural palatability enhancers ensure an active first feeding response.
1.2, 1.5, 2, 2.5, and 3 mm	47	18	Mid-level energy fish feed for moderate or controlled growth; includes alternative ingredients to reduce cost; contains enhanced vitamin pack and pigment to promote healthy fish and natural coloration.
4 mm 6 mm 9 mm	45 43 40	24 24 24	High-energy trout and steelhead diet designed to give maximum growth and the lowest feed conversion rates. Pigment is included in sizes 6 and 9 mm to promote natural coloration.
		RANGEN	
#0, #1, and #2	52	16	Especially formulated for first feeding fry and fingerling; nearly free of dust and fines; high levels of quality fish and animal protein; utilizes marine oils, beta glucans, pigments, and high vitamin levels, including stabilized vitamin C.
#3 and #4	45	15	Nutritionally complete and balanced; main components are high quality fish and animal proteins; finely ground to ensure superior digestibility and that fish receive full complement of nutrients.
3/32”, 1/8”, and 5/32”	40	12	Sinking pellets manufactured by steam pelleting; suitable for fish from 15 grams to 900 grams or more.
3/16” and 1/4”	40	10	Includes high levels of vitamins, minerals, and antioxidants; unique ingredients and balanced formulation proven to aid in the well being of brood fish and viable egg production; extrusion process allows slow sinking or floating pellets.

Table A3.1.2. Bio Oregon suggested feeding rate (% BW/d) by feed size, fish size, and at a temperature of 54°F (from Fetherman et al. 2019).

Feed Size	Count per Pound	Length (in)	Weight (g)	Feeding Rate
#0	3000-570	Hatch-1.7	0.15-0.8	3.3
#1	570-300	1.7-2.1	0.8-1.5	3.1
#2	300-150	2.1-2.6	1.5-3.0	3.0
1.2 mm	150-90	2.6-3.1	3.0-5.0	2.9
1.5 mm	90-60	3.1-3.5	5.0-8.0	2.7
2 mm	60-25	3.5-4.6	8.0-18	2.4
2.5 mm	25-11	4.6-6.0	18-40	1.9
3 mm	11-6	6.0-7.4	40-75	1.4
4 mm	> 6	> 7.4	> 75	1.0

Table A3.1.3. Rangen suggested feeding rate (% BW/d) by feed size, fish size, and at a temperature of 53°F (from Fetherman et al. 2019).

Feed Size	Count per Pound	Length (in)	Weight (g)	Feeding Rate
#0	< 1,200	< 1.3	< 0.4	5.4
#1	1,200	1.3	0.4-0.8	5.4
#2	600	1.5	0.8-1.5	4.5
#2	300	2.0	1.5-2.3	3.9
#3	200	2.3	2.3-4.5	3.5
#3	100	2.8	4.5-6.0	2.9
#4	80	3.0	6.0-8.0	2.7
#4	60	3.5	8.0-11.0	2.5
3/32”	40	4.0	11.0-15.0	2.3
3/32”	30	4.5	15.0-21.0	2.2
3/32”	22	4.8	21.0-30.0	2.0
1/8”	15	5.5	30.0-38.0	1.8
1/8”	12	6.0	38.0-50.0	1.7
5/32”	9	6.5	50.0-76.0	1.6
5/32”	6	7.5	76.0-114.0	1.4
3/16”	4	8.5	114.0-151.0	1.3

APPENDIX 3.2: FULTON'S CONDITION FACTOR MODEL

y_i : Fulton's condition factor for each hatchery trough

μ_i : Mean condition factor for each hatchery trough based on Density, Feed, and Density*Feed

σ : Standard deviation of the mean condition factor

$\mathrm{Denisty}_i$: Density (Low, 0; High, 1)

Feed_i : Feed type (Rangen, 0; Bio Oregon, 1)

S_0 : Mean condition when there is no Density or Feed

S_1 : Regression coefficient associated with Density

S_2 : Regression coefficient associated with Feed

S_3 : Regression coefficient associated with the Density by Feed interaction

$$\mathrm{y}_i \sim \mathrm{normal}(\mu_i, \sigma^2)$$

$$\mu_i = \mathrm{S}_0 + \mathrm{S}_1\mathrm{Density}_i + \mathrm{S}_2\mathrm{Feed}_i + \mathrm{S}_3(\mathrm{Density}_i * \mathrm{Feed}_i)$$

$$\sigma \sim \mathrm{uniform}(0, 100000)$$

$$\mathrm{S}_0 \sim \mathrm{gamma}(2, 1)$$

$$\mathrm{S}_1 \sim \mathrm{normal}(0, 1000)$$

$$\mathrm{S}_2 \sim \mathrm{normal}(0, 1000)$$

$$\mathrm{S}_3 \sim \mathrm{normal}(0, 1000)$$

Posterior

$$[\mathbf{S}, \mathrm{S}_0, \sigma \mid \mathbf{y}] \propto \prod_{i=1}^{n} [y_i \mid \mathrm{S}_0, \mathbf{S}, \sigma][\sigma]\ [\mathrm{S}_0]\ [\boldsymbol{S}]$$

APPENDIX 3.3: LENGTH MODEL

y_i : Length for each hatchery trough

μ_i : Mean length for each hatchery trough based on Density, Feed, and Density*Feed

σ : Standard deviation of the mean length

$\mathrm{Denisty}_i$: Density (Low, 0; High, 1)

Feed_i : Feed type (Rangen, 0; Bio Oregon, 1)

S_0 : Mean length when there is no Density or Feed

S_1 : Regression coefficient associated with Density

S_2 : Regression coefficient associated with Feed

S_3 : Regression coefficient associated with the Density by Feed interaction

$$\mathrm{y}_i \sim \mathrm{normal}(\mu_i, \sigma^2)$$

$$\mu_i = \mathrm{S}_0 + \mathrm{S}_1\mathrm{Density}_i + \mathrm{S}_2\mathrm{Feed}_i + \mathrm{S}_3(\mathrm{Density}_i * \mathrm{Feed}_i)$$

$$\sigma \sim \mathrm{uniform}(0, 100000)$$

$$\mathrm{S}_0 \sim \mathrm{gamma}(100, 1)$$

$$\mathrm{S}_1 \sim \mathrm{uniform}(-1000, 1000)$$

$$\mathrm{S}_2 \sim \mathrm{uniform}(-1000, 1000)$$

$$\mathrm{S}_3 \sim \mathrm{uniform}(-1000, 1000)$$

Posterior

$$[\mathbf{S}, \mathrm{S}_0, \sigma \mid \mathbf{y}] \propto \prod_{i=1}^{n} [y_i \mid \mathrm{S}_0, \mathbf{S}, \sigma][\sigma]\ [\mathrm{S}_0]\ [\boldsymbol{S}]$$

APPENDIX 3.4: WEIGHT MODEL

y_i : Weight for each hatchery trough

μ_i : Mean weight for each hatchery trough based on Density, Feed, and Density*Feed

σ : Standard deviation of the mean weight

$\mathrm{Denisty}_i$: Density (Low, 0; High, 1)

Feed_i : Feed type (Rangen, 0; Bio Oregon, 1)

S_0 : Mean weight when there is no Density or Feed

S_1 : Regression coefficient associated with Density

S_2 : Regression coefficient associated with Feed

S_3 : Regression coefficient associated with the Density by Feed interaction

$$\mathrm{y}_i \sim \mathrm{normal}(\mu_i, \sigma^2)$$

$$\mu_i = \mathrm{S}_0 + \mathrm{S}_1 \mathrm{Density}_i + \mathrm{S}_2 \mathrm{Feed}_i + \mathrm{S}_3(\mathrm{Density}_i * \mathrm{Feed}_i)$$

$$\sigma \sim \mathrm{uniform}(0, 100000)$$

$$\mathrm{S}_0 \sim \mathrm{gamma}(10, 1)$$

$$\mathrm{S}_1 \sim \mathrm{uniform}(-1000, 1000)$$

$$\mathrm{S}_2 \sim \mathrm{uniform}(-1000, 1000)$$

$$\mathrm{S}_3 \sim \mathrm{uniform}(-1000, 1000)$$

Posterior

$$[\mathbf{S}, \mathrm{S}_0, \sigma \mid \mathbf{y}] \propto \prod_{i=1}^{n} [y_i \mid \mathrm{S}_0, \mathbf{S}, \sigma][\sigma]\ [\mathrm{S}_0]\ [\boldsymbol{S}]$$

APPENDIX 3.5: HEPATOSOMATIC INDEX (HSI) MODEL

y_i : HSI for each hatchery trough

μ_i : Mean HSI for each hatchery trough based on Density, Feed, and Density*Feed

σ : Standard deviation of the mean HSI

$\mathrm{Denisty}_i$: Density (Low, 0; High, 1)

Feed_i : Feed type (Rangen, 0; Bio Oregon, 1)

S_0 : Mean weight when there is no Density or Feed

S_1 : Regression coefficient associated with Density

S_2 : Regression coefficient associated with Feed

S_3 : Regression coefficient associated with the Density by Feed interaction

$$\mathrm{y}_i \sim \mathrm{normal}(\mu_i, \sigma^2)$$

$$\mu_i = \mathrm{S}_0 + \mathrm{S}_1\mathrm{Density}_i + \mathrm{S}_2\mathrm{Feed}_i + \mathrm{S}_3(\mathrm{Density}_i * \mathrm{Feed}_i)$$

$$\sigma \sim \mathrm{uniform}(0, 100000)$$

$$\mathrm{S}_0 \sim \mathrm{gamma}(2, 1)$$

$$\mathrm{S}_1 \sim \mathrm{uniform}(-1000, 1000)$$

$$\mathrm{S}_2 \sim \mathrm{uniform}(-1000, 1000)$$

$$\mathrm{S}_3 \sim \mathrm{uniform}(-1000, 1000)$$

Posterior

$$[\mathbf{S}, \mathrm{S}_0, \sigma \mid \mathbf{y}] \propto \prod_{i=1}^{n} [y_i \mid \mathrm{S}_0, \mathbf{S}, \sigma][\sigma]\ [\mathrm{S}_0]\ [\boldsymbol{S}]$$

APPENDIX 3.6: CAPTURE-RECAPTURE MODEL

$$\mathrm{y}_{it} \sim \text{Bernoulli}(p_t * z_{it})$$

$$\mathrm{z}_{it} \sim \text{Bernoulli}(\phi_i^{\frac{2}{52}} * z_{i(t-1)}) \quad \text{for } t = 2,\ 3,\ 4,\ 5$$

$$\mathrm{z}_{it} \sim \text{Bernoulli}(\phi_i^{\frac{22}{52}} * z_{i(t-1)}) \quad \text{for } t = 6$$

$$\mathrm{z}_{it} \sim \text{Bernoulli}(\phi_i^{\frac{12}{52}} * z_{i(t-1)}) \quad \text{for } t = 7,$$

$$\mathrm{z}_{it} \sim \text{Bernoulli}(\phi_i^{\frac{10}{52}} * z_{i(t-1)}) \quad \text{for } t = 8$$

$$(\phi_i) = \text{inverse logit}(\beta_0 + \beta_{\text{Density}} * (\text{Density}_i) + \beta_{\text{Feed}} * (\text{Feed}_i) + \beta_{\text{Density*Feed}} * (\text{Density}_i * \text{Feed}_i))$$

$$p_t \sim \text{beta}(1, 1)$$

$$\beta_0 \sim \text{normal}(0, 1.96)$$

$$\beta_{\text{Density}} \sim \text{normal}(0, 1.96)$$

$$\beta_{\text{Feed}} \sim \text{normal}(0, 1.96)$$

$$\beta_{\text{Density*Feed}} \sim \text{normal}(0, 1.96)$$

Posterior

$$(\phi_i) = \text{inverse logit}(\beta_0 + \beta_{\text{Density}} * (\text{Density}_i) + \beta_{\text{Feed}} * (\text{Feed}_i) + \beta_{\text{Density*Feed}} * (\text{Density}_i * \text{Feed}_i))$$

$$[\mathrm{z}, \boldsymbol{\beta}, \boldsymbol{p} \mid \mathbf{y}] \propto \prod_{i=1}^{n} \prod_{t=2}^{8} [y_{it} \mid p_t * z_{it}] \prod_{t \in 2,3,4,5} [z_{it} \mid z_{i(t-1)}, \phi_i^{\frac{2}{52}}] \prod_{t \in 6} [z_{it} \mid z_{i(t-1)}, \phi_i^{\frac{22}{52}}] \prod_{t \in 7} [z_{it} \mid z_{i(t-1)}, \phi_i^{\frac{12}{52}}] \prod_{t \in 8} [z_{it} \mid z_{i(t-1)}, \phi_i^{\frac{10}{52}}] [\boldsymbol{p}] [\boldsymbol{\beta}]$$