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The Effect Of Slow Release Cortisol Implant On Humoral Immune Responses And Infection Prevalence Following Experimental Challenge With Flavobacterium Psycrophilum In Rainbow Trout (Oncorhynchus Mykiss)

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APPROVAL PAGE

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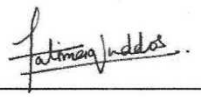

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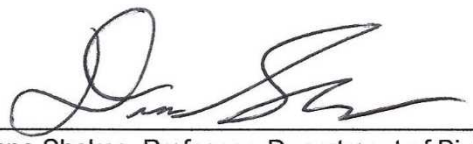

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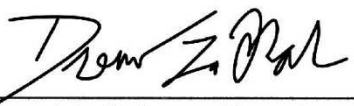

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COMPLIANCE PAGE

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ABSTRACT

Pacific salmon migrate long distances to spawn as part of their life cycle. During this journey from sea to their natal stream, they undergo major endocrine, physiological and immune changes. Cortisol, the primary stress hormone, gradually increases during the journey. Persistent high cortisol levels have deleterious health effects, including suppression of the antibody response. However, pathogens encountered during their journey may stimulate antibody responses to overcome the infection. My main research question focuses on how salmonids balance the immunosuppressive effects of high cortisol levels with activation of the antibody response. A recent field study from our lab showed a transient increase in abundance of B cells during the spawning run which is suggestive of activation of the immune system during this journey. However, our field study had too many confounding variables. In this study, we investigated the activation of the antibody response under conditions of elevated levels of cortisol in rainbow trout under laboratory-controlled conditions. We looked at the effects of a) cortisol alone, b) fish pathogen *Flavobacterium psycrophilum (Fp)* alone and c) combined cortisol and *Fp* challenge on the gene expression of immunoglobulins IgM and IgT using qPCR. We have found that cortisol suppresses the IgM response in the spleens of *Fp*-susceptible line but not in *Fp*-resistant line of Rainbow trout. No significant effects on B cell development where observed in the anterior kidney. Taken together, our data suggest that the antibody response in *Fp*-resistant rainbow trout is less sensitive to increased cortisol levels compared to *Fp*-susceptible fish, confirming our hypothesis that *Fp*-resistant fish have in some way evolved to manage stress more successfully.

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ACKNOWLEDGEMENTS

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This M.S. is dedicated to my parents and my husband, Shah Rukh, who have helped me countless times along the way. They are my rocks; I can always rely on them for help and support.

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Abbreviation	**Meaning**
R Line	Resistant Line
S Line	Susceptible Line
Fp	*Flavobacterium psychrophilum*
RBT	Rainbow Trout
memHCmu	Membrane heavy chain mu
secHCmu	Secreted heavy chain mu
memHCtau	Membrane heavu chain tai
secHCtau	Secreted heavy chain tau
MU Ratio	secHCmu/memHCmu
TAU Ratio	secHCtau/memHCtau
L	Line
TG	Treatment Group
DPI	Days post infection
ANOVA	Analysis of variance
PBL	Peripheral blood lymphocyte

Chapter 1

The effect of slow release cortisol implant on humoral immune responses and infection prevalence following experimental challenge with Flavobacterium psycrophilum in Rainbow trout (*Oncorhynchus mykiss*)

1. Introduction

Stress has become a well-known and recognized aspect of life. Several studies have examined the relationship between stress and immune response in higher vertebrates. Chronic stress suppresses immune response (Dhabhar, 2014) but, acute stress has been shown to have enhancing effects, at least in some studies (Dhabhar, 2014; Pedersen and Toft, 2000). Like higher vertebrates, fish undergo stress from crowding, handling, moving, and disease, and the subsequent stress responses affect their immune system. Salmonids, specifically *Oncorhynchus mykiss* and *Oncorhynchus nerka*, have severely elevated levels of cortisol, primary stress hormone, as part of their life cycle. During migration from ocean to the spawning sites, there is a gradual increase in the levels of cortisol which peaks before the spawning stage (Baker and Vynne, 2014). However, they are still able to survive this rigorous journey of swimming upstream and battling pathogens.

To examine the relationship between stress and immunity in salmonids, Smith and Zwollo (manuscript submitted) studied the immune system of sockeye salmon during the return spawning journey from the ocean to their natal spawning ground in the Kenai River, Alaska. Smith's study used flow cytometry to determine the abundance of anterior kidney cells in different stages of development during the return journey. The abundance of both (im)mature B cells, reflective of the production of B cells, as well as the plasma cells, reflective of the activation of B cells, transiently increased during the

return journey. The study also examined myeloid cell populations but did not report any significant changes.

Along with abundance of different types of B cells, Smith's study also measured Spleen index. Spleen Index is the measure of spleen weight relative to the body weight of the fish and is a unitless entity which can be compared across fish regardless of their body weight. Interestingly from an immunologist's standpoint, spleen index can also reflect the extent of activation of the immune system, particularly the antibody response(ref). It was reported in Smith's study that in *Oncorhynchus nerka,* spleen index mirrored the transient increase of the abundance of B cells. Further as expected, both the spleen index and the B cell abundance was found to be lowest at the spawning sites, as fish enter the final stage of their life cycle.

Several studies, including Smith's, have investigated pathogen loads in migrating salmonids during the journey and at the spawning sites. Smith's data indicated that fish at their spawning sites had the highest pathogen prevalence and intensity of Infectious hematopoietic necrosis virus (IHNV), compared to fish further downstream, on the same river and during the same run. This is supported by the finding of a positive correlation between pathogen presence (IHNV) and distance from the mouth of the river (Smith & Zwollo). Another study investigated different pathogens at different spawning sites during the same run, the Kenai run. Both IHNV and *Flavobacterium psycrophilum* (*Fp*) were higher farther from the mouth of the river (Chappell et al., 2017). These data suggest that pathogen loads are highest at the spawning grounds.

Twardek et al (Twardek et al., 2019) measured infectious agents including *Fp* before and after a waterfall in steelhead trout from gill tissues and reported that fish sampled below the waterfall had higher pathogen load as compared to fish sampled upstream from the waterfall. Contrary to Smith and Chappell, they concluded that fish

with higher pathogen loads are less likely to overcome the upstream migratory barriers such as a hydraulically challenging reach. In combination with the observations that pathogen loads are highest at the spawning grounds, this raises (unanswerable) questions about the dynamics of pathogens and immune responses during this final stage of the life cycle of the salmon.

Smith's study was exploratory in nature; thus, it is difficult to establish cause and effect. The results suggest that sockeye salmon undergo antibody activation during the return migration journey, despite chronic stress and/or high cortisol conditions. However, there are other factors during the return journey that might enhance the immune system activation such as catecholamines, growth hormones and thyroid hormones reviewed in (Zwollo, 2018). These results are of special interest because they suggest that the immune system can overcome the suppressive effects of cortisol when challenged by pathogens. However, looking at the data in Smith's study, two possible outcomes can be inferred:

1) The activation of immune system during the journey helped salmon combat encountered pathogens effectively: Antibody response returned to the baseline levels when they reached the spawning site.
2) Salmon that activated their immune system after encountering pathogens were unable to fight the infection which led to early mortality. The only subset of migrating salmon that reached the spawning grounds were those that were able to combat the pathogens without activating the antibody response.

In the current study, the possible outcome will be determined by the pathogen load in individuals with an activated immune system (antibody response), as compared to fish that don't show immune activation. **Hence, my research aims to test the following two hypotheses under controlled lab conditions 1) the immune system of**

migrating salmonids can be activated during pathogen challenge despite high cortisol levels and 2) the activation of the immune system correlates with higher pathogen load, which is detrimental to survival.

Rainbow trout, *Oncorhynchus mykiss*, has been selected as the animal model for this study for several important reasons. First, *Oncorhynchus nerka* and *Oncorhynchus mykiss* belong to the same genus and both are anadromous teleost fish. Further, it has been established in our lab that same antibody reagents work for both species, supportive of their similarity in immune components. Hence, the results obtained from this study will be comparable to the Smith study.

1.1 Immune system of Rainbow trout

Rainbow trout (RBT) is among the most studied teleost species and is widely used in aquaculture. This can be attributed to its ability to survive and reproduce under a vast range of conditions. RBT can withstand temperatures ranging from 0 to 27 degree Celsius and grow rapidly, achieving 7-10 kg in 3 years. This robustness, in part, can be attributed to its well-developed immune system which is similar to that of mammals. The innate immune system provides the first line of defense and includes physical barriers, inflammation, innate immune cells including macrophages and neutrophils and the complement system. If a pathogen overcomes the initial innate defense mechanisms and is not cleared out, it leads to the activation of an adaptive immune response which is geared toward that specific pathogen. Teleost were the first vertebrates to develop adaptive immunity; mounting antibody responses to foreign antigens. B-lineage lymphocytes produce specific antibodies, comprising humoral immunity.

Salmonids possess B- cell lymphocytes very similar to those in mammalian species despite the absence of bone marrow and lymph nodes. The anterior kidney

functions as a hematopoietic site analogous to bone marrow in mammals (Hansen and Zapata, 1998). Multiple studies have suggested that while B cells develop in the anterior kidney, activation of B cells occurs primarily after cells have migrated to the spleen (Bromage et al., 2004; Zwollo et al., 2005, 2008). After activation in the spleen, B cells proliferate and differentiate into transitional (T-PC) and/or mature (M-PC) plasma cells (Barr et al., 2011). Activation of B-cells might lead to enlargement of the spleen with a larger spleen index, although this specific linkage has not been reported yet. Hadidi et al reports a higher survival correlated with a higher spleen index after an experimental challenge (Hadidi et al., 2008).

In continuation with the anterior kidney's functionally analogous role as the mammalian bone marrow, a small subset of immunoglobulin-secreting plasmablasts or T-PCs relocate to the anterior kidney where they can differentiate into long-lived plasma cells (LLPCs). Such cells keep secreting antibodies even in the absence of antigens for months and may continue doing so for years (Bromage et al., 2004; Kaattari et al., 2005; Ye et al., 2011; Ma et al., 2013).

ISC's secrete two distinct types of antibodies in teleost species including rainbow trout; IgM and IgT (Hansen et al., 2005; Warr et al., 1979; Zhang et al., 2010). IgM is essential for systemic immunity whereas IgT plays major roles in mucosal immunity. Immature B cells have membrane IgM and IgT on their cell surface, known as resting B-cells. After they encounter a pathogen, a subset of such cells, those with high affinity for the pathogen, will become activated, proliferate and start secreting either IgM or IgT, known as plasmablasts. These cells can further differentiate into plasma cells, which can secrete much higher amounts of secreted immunoglobulins compared to plasmablasts. The secreted antibodies function as a clearance mechanism of the pathogen and help the body restore homeostasis.

Transcript levels of heavy chain (HC) genes for both mu and tau (HCmu and HCtau) are a good measure for both membrane(mem) and secreted(sec) forms of IgM and IgT antibody proteins. A review paper examined the pros and cons of measuring mRNA transcripts through gene expression studies vs. the actual protein through flow cytometry or protein expression studies (Liu et al., 2016). They report a strong correlation between mRNA and protein levels in steady state cells (Liu et al., 2016). Furthermore, studies from our lab and others have shown a strong correlation between antibody protein and mRNA gene expression levels (Nakanishi et al., 1984; Gry et al., 2009; Zwollo et al., 2017) . Therefore, the ratio of secreted/membrane immunoglobulin (SecHCmu/memHCmu or SecHCtau/memHCtau) is a good predictor of the activation state of the B cells.

1.2 Fish response to stress and pathogens

The biological stress response has evolved as a mechanism to help organisms survive. It redirects how an organism normally uses its energy reserves and prioritizes the energy expenditure towards the required physiological systems. Cortisol, a glucocorticoid, is the primary stress hormone in teleost fish, and is regulated by the Hypothalamic-Pituitary-Interrenal (HPI) axis. The HPI axis ensures that not only the body is well prepared to face and respond quickly to stressful stimuli but also enables it to return quickly to baseline levels of cortisol to resume normal body functions. Acute stress prepares the body for physiological fight or flight responses. The body is able to recover well after an acute stressful event. However, chronic stress has deleterious health effects including suppression of immune system reviewed in (Dhabhar, 2014). High levels of cortisol for longer durations such as under chronic stress can lead to cortisol insensitivity or "resistance" (Zeitzer et al., 2016). Moreover, it is known that

dysregulation of cortisol is linked to impairment of physiological systems such as metabolism, physical activity, and immune functioning in vertebrates and higher animals.

Cortisol induces suppression of several key mechanisms of the antibody response, such as B cell development, B cell activation (Tripp et al., 1987), and B cell proliferation (Verburg-van kemenade et al., 1999). In carp, a comparison of B lymphocytes isolated from different immune tissues treated with cortisol showed that it induced apoptosis in B cells, and also suppressed proliferation at the plasmablast and developmental stages (Verburg-van kemenade et al., 1999). Another study showed that apoptosis was specifically induced in activated B cells (Weyts et al., 1998). Similarly Espelid et al, showed that the number of Ig positive cells dramatically reduced after a cortisol injection in Atlantic salmon (Espelid et al., 1996). Another study in juvenile rainbow trout showed reduced circulating lymphocytes (peripheral blood lymphocytes or PBLs) after a 10 week diet with cortisol, however the study does not specify the type of lymphocytes (Barton et al., 1986). Lymphocytopenia was also observed in Brown trout following cortisol administration by Pickering (Pickering, 1984). A slow release cortisol implant study in rainbow trout showed strong modulation of the gene expression of different components of innate immune system including the complement system (Cortés et al., 2013).

1.3 Flavobacterium psycrophilum (Fp)

In the current study, pathogen exposure is performed by intra-muscular injection of a fish pathogen; *Fp. Fp* is the etiological agent for bacterial cold-water disease (BCWD) also known as rainbow trout fry syndrome. It is named as such because it causes outbreaks at water temperatures below 15 C, mostly in the earlier part of life cycle (fry stage). In addition, it is a common pathogen encountered in freshwater streams by migrating salmonid species (Chappell et al., 2017; Twardek et al., 2019).

Rainbow trout is the most susceptible at the fry stage because adaptive immune system is not completely developed, hence they rely only on the innate immune response. However, its pathogenicity is not limited to rainbow trout; it can infect *Oncorhynchus nerka* and other salmon in the *Oncorhynchus* genus. In wild as well as in hatchery fish *Fp* causes considerable losses in aquaculture worldwide (Nematollahi et al., 2003; E. Barnes and L. Brown, 2011)

Fp is a flexible, rod-shaped gram-negative bacterium that can be cultivated on nutrient poor media such as tryptone yeast salt extracts (TYES). It is an active growing bacterium and forms bright yellow raised colonies. Optimal growth rate of this bacterium is achieved at 15-17 degrees centigrade and it has higher pathogenicity at lower water temperatures.

The mechanism through which *Fp* infects fish has yet to be elucidated. However, it has been shown that *Fp* adheres to gills and skin of the host without causing infection and enters when the immune system is weakened or suppressed, and/or through skin lesions. Hence it is thought to be an opportunistic pathogen (Nematollahi et al., 2003; Henriksen et al., 2015). Different routes of exposure have been used including intramuscular, oral, anal, or peritoneal injection to infect fish with *Fp*. The most commonly used method is through intramuscular injections, which causes infection in ~80% of the fish. Common symptoms of *Fp*-infection include erratic swimming, muscle degradation, internal bleeding, swelling and injury around the mouth, degradation of gills, anemia, vertebral deformities and skin discoloration and darkening (Castillo et al., 2012).

Several studies have reported on immune effects of *Fp* challenge in rainbow trout. In a comparative transcriptomic study, innate immune genes that were affected by *Fp* infection included complement factors h, h-like, c3 and c9, all of which were upregulated in *Fp* challenged trout (Langevin et al., 2012; Marancik et al., 2015).

Acquired immune genes including HCmu, HCtau, cd3, cd4, cd8 and mhcII were slightly upregulated after *in vivo Fp* challenge in whole fry (Marancik et al., 2015). However, when specific immune organs were studied after *in vivo Fp* challenge (instead of whole body), the expression of both secreted and membrane HCmu and HCtau were downregulated in three major immune sites, including anterior kidney, spleen and blood (Zwollo et al., 2017). Incubation of *Fp* with serum containing only antibodies from fish infected with *Fp* only slightly decreased the number of bacterial cells, which might suggest that humoral response is not effective by itself (Nematollahi et al., 2003). (Decostere et al., 2001) looked at splenic cells with phagocytic activity in experimentally infected rainbow trout fry and reported viable bacteria inside these phagocytes. However, in the same experiment, adult rainbow trout did not yield any viable bacterial cells. Presumably, *Fp* can survive inside macrophages, thereby preventing the host to clear out the pathogen (Decostere et al., 2001; Nematollahi et al., 2003).

As aquaculture is rapidly replacing capture fisheries, disease outbreaks are one of the major reasons for reduced production and increased mortality of fish. The most common treatment to counter such outbreaks is the use of antibiotics. As we become more and more aware of consuming antibiotic treated food products and its harmful effects, there is a need to look for alternate treatments. Currently, no commercial vaccine is available against *Fp* although active research is underway. Other alternatives include prophage treatment to combat *Fp* infection. Some studies have reported and characterized different phages, such as Stenholm et al characterized 22 *F. psychrophilum* phages from Danish rainbow trout farms and Castillo et al characterized 12 phages isolated from Chilean salmonid aquaculture. Stenholm et al reported that most phages were either only effective for their *Fp* host strains, the strain they were originally isolated from whereas Castillo et al also reported high variation towards

susceptibility on the level of individual strains (up to 10^7-fold differences) to specific phages (Stenholm et al., 2008; Castillo et al., 2012). In other words, the phage response is not a practical solution yet as it seems to be host strain specific.

One alternative solution has been highly successful: selective genetic breeding to generate a *Fp*-resistant line (R-line) of rainbow trout (Silverstein et al., 2009; Leeds et al., 2010). Although the genetic basis of the resistance is still unknown, it has been shown that R-line fish have a higher spleen index (Hadidi et al., 2008), and that resistant fish have lower pathogen loads after *Fp*-challenge compared to a control (Susceptible line) line of RBT (Marancik et al., 2014, 2015). Several studies have reported on the differential regulation of immunoglobulin expressing genes in R and S-lines. Marancik reported (Marancik et al., 2015) higher igm gene transcript levels in resistant line compared to the susceptible-line post experimental challenge with *Fp*. A previous study from our lab (Zwollo et al., 2017) has shown that IgT+ cells are more abundant in naïve R-line as compared to S-line, suggesting a stronger mucosal immunity. However, (Langevin et al., 2012) did not report any significant changes in igm and igt gene expression in anterior kidney of naïve or experimentally challenged rainbow trout.

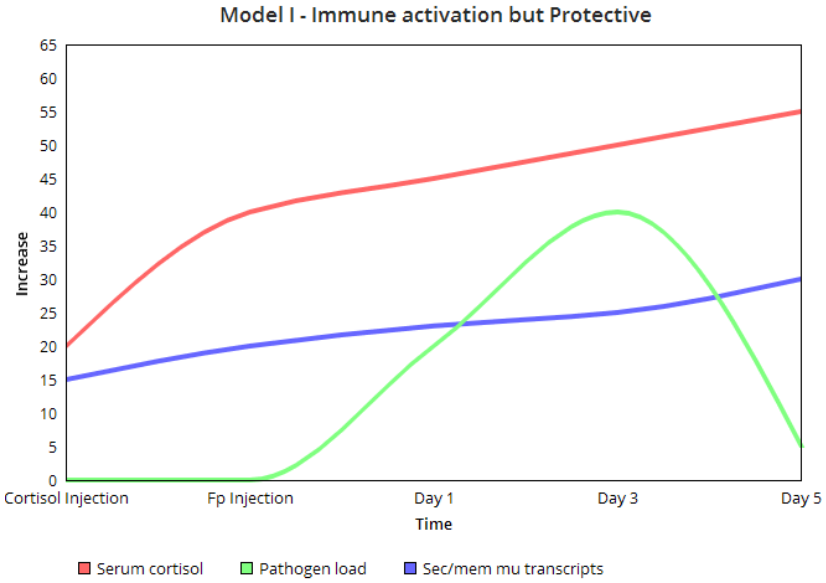
2. Conceptual framework and Research Questions

So far, studies have investigated immune responses to either the *Fp* pathogen, or the stress hormone cortisol. In this study, I aim to investigate the combined effect of cortisol and *in vivo Fp* challenge on the antibody response in rainbow trout. Interactions with pathogen typically stimulate the antibody response, while elevated levels of cortisol are likely to suppress the immune system. Hence, my main research question is how the immune response to *in vivo Fp*-challenge will be affected by experimentally increased cortisol levels, and further, whether *Fp*-resistant fish will be able to maintain their resistance under such conditions. In the current study, trout were experimentally

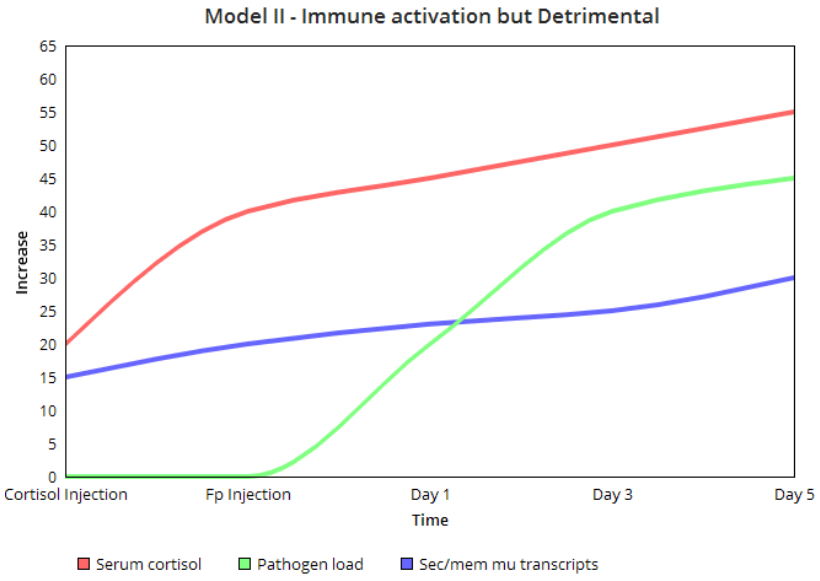
challenged with *Fp* 5 days after an intraperitoneal treatment of cortisol implants. Anterior kidney, spleen, and posterior kidney cells were collected on Day 1, 3 and 5 post *Fp*-challenge, and gene expression of secreted and membrane forms of HCmu and HCtau determined

Considering the previous findings and combining the suppressive effects of cortisol with the stimulatory effects of a pathogen, I propose two possible models. These models can form the basis of whether the salmonids have evolved in some way to manage the immune-suppressive effects of cortisol and if the immune response is protective or detrimental during high cortisol serum levels.

Model 1: Elevated serum cortisol levels post *Fp* challenge correlate with higher ratio of sec/mem immunoglobulins, and lower pathogen loads in a fish. This would lead us to hypothesize that salmonids are able to lower their pathogen loads by inducing a successful antibody response despite high cortisol levels.



Model 2: Elevated serum cortisol levels post *Fp* challenge correlate with higher pathogen loads and higher ratio of sec/mem immunoglobulins. Stronger antibody responses are not able to lower pathogen load which suggests a detrimental immune response.



2.1 Research Questions

1. Does exposure to cortisol implants affect the Ig response in rainbow trout after challenge by *Flavobacterium psycrophilum* (*Fp*)?

2. Are there differences in expression of immunoglobulin expression and pathogen load in a *Fp* resistant line of RBT compared to a susceptible (*Fp*S) control line, after *Fp* challenge in fish containing cortisol implants?

3. Is the Ig response to *Fp* challenge in fish Injected with slow release cortisol implants protective or detrimental to the health the fish as reflected by *Fp*

pathogen load? (Does pathogen load correlate with sec/mem ratio of HCmu and HCtau in cortisol treated, *Fp* challenged fish?)

In the current study, we aim to evaluate how chronically elevated levels of cortisol affect the immunoglobulin response. Several studies (Ising and Holsboer, 2006; Miller et al., 2008; Morey et al., 2015) have investigated genetic alterations in response to chronic stress in humans. Patients suffering from terminal or auto-immune diseases or caregivers of such patients can be under chronic stress which affects their antibody response. One study reports that although cortisol levels of caregivers are similar to control group, the downregulatory signals of cortisol to suppress inflammation are not 'heard' by the cells leading to the continuous inflammatory state (Miller et al., 2008). Our study can help form the basis of future studies relevant for ameliorating the effects of chronic stress.

In addition, a clearer understanding of how stress and immunity are linked in fish species may translate to improvements in global seafood sustainability. Seafood makes up a big portion of all the food consumed in the world and it is expected to increase. Aquaculture, production of high densities of fish in hatcheries, is part of the solution to feeding the global population. Outbreaks caused by pathogens and fish health in general have arisen as a major problem in aquaculture in the past decade. Further, overfishing has brought some species on the brink of extinction. My research, ultimately, can influence aquaculture and may help in getting some species off the endangered list. The study will also be helpful for managing aquaculture policies in the pacific Northwest.

Chapter 2

A Bacterial Cold-Water Disease-Resistant Line of Rainbow Trout is Less Sensitive to Cortisol Implant-Induced Changes in IgM Response as Compared to a Susceptible (Control) Line

A BCWD-Resistant Line of Rainbow Trout is Less Sensitive to Cortisol Implant-Induced Changes in IgM Response as Compared to a Susceptible (Control) Line

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ABSTRACT

In salmonids, stress responses increase serum cortisol levels which in turn suppress immune responses and increase disease susceptibility. *Flavobacterium psychrophilum (Fp)* is the causative agent of BCWD. Selective genetic breeding has generated a *Fp*-resistant line (*Fp*-R-line) of rainbow trout and was used here to investigate potential differences in immunoglobulin response after a combined treatment of cortisol and *Fp,* compared to a (susceptible or *Fp*-S-line) control. Expression of both the membrane and secreted splice forms of immunoglobulin heavy chains mu and tau were determined in spleen and anterior kidney. Results suggest that an earlier IgM response is a determining factor in differential disease progression between the R- and S-line after *Fp*-challenge. Further, cortisol treatment equalizes IgM response at the early stage of infection in both lines, followed by reduced *Fp*-pathogen load exclusively in R-line fish. Our results reveal a delayed and exacerbated IgM response in S line fish after cortisol implant, indicative of a detrimental cycle of both high, sustained IgM responses and pathogen loads.

INTRODUCTION

Flavobacterium psychrophilum (*Fp*), the causative bacterial agent for bacterial cold-water disease (BCWD), is a major source of disease in salmonid species, in the wild and aquaculture, including rainbow trout (RBT) (Starliper, 2011). *Fp* significantly affects production in salmonid hatcheries and aquaculture facilities worldwide and causes mortality in wild migrating salmon (Starliper, 2011; Twardek et al., 2019). Selective genetic breeding to generate *Fp*-resistant lines (*Fp*-R-line) of RBT has been a successful approach to fight BCWD (Wiens et al., 2013a, 2018). Further, having a *Fp*-R-line of RBT has provided an important tool for studying the immunological modes of disease protection (Marancik et al., 2015; Zwollo et al., 2015). Although the genetic basis of the resistance is still under investigation, it has been shown that R-line fish often have a higher spleen index (Hadidi et al., 2008; Wiens et al., 2013b; Zwollo et al., 2017), and lower pathogen loads after *Fp*-challenge as compared to a control (Susceptible line; S-line) line of RBT (Marancik et al., 2015; Wiens et al., 2013b; Zwollo et al., 2017). The spleen is a critical secondary immune organ where B-cells are activated and terminally differentiate into antibody-secreting cells [plasmablasts, transitional plasma cells (T-PCs) and plasma cells]. Spleen index defines spleen weight relative to the bodyweight of a fish and has been linked with increased survival after *Fp*-challenge (Hadidi et al., 2008).

The humoral immune system likely plays a role in *Fp* resistance in RBT, but the mechanisms are only partially understood. The two major Ig classes well-studied in RBT are IgM and IgT (a third class is IgD); IgM is the most prevalent antibody isotype and is thought to be involved in both systemic and mucosal defense that make up a major component of humoral immunity (Castro et al., 2013; Warr et al., 1979). On the other hand, IgT is the functional equivalent of mammalian IgA, and as such plays an essential role in mucosal immunity. A study by Salinas et al. (Salinas et al., 2011) concluded that

IgT is expressed mutually exclusive of IgM. IgT^+ B cells have similar phagocytic and microbicidal activities as IgM. Upon microbial stimulation *in vitro*, IgT^+ and IgM^+ B cells isolated from anterior kidney of RBT secrete high amounts of immunoglobulins (Igs) (Salinas et al., 2011; Zhang et al., 2010).

We have shown previously that naïve R-line fish had lower abundance of IgM^+ and IgM^{++} cells in their immune organs compared to S-line fish (Zwollo et al., 2015). In contrast, the abundance of IgT^+ cells was significantly higher, both in naïve and *Fp*-challenged R-line fish, as compared to S-line fish. Of interest, higher abundance of IgT^+ B cells and mRNA expression of the secreted form of Ig heavy chain tau (secHCtau) correlated with lower *Fp* loads in challenged fish, while in the anterior kidney, IgM^+ B cell abundance correlated with increased *Fp* loads. Together, these results suggested that IgT^+ B lineage cells may have a protective function in the immune response to *Fp* (Zwollo et al., 2017). However, a whole body transcriptomic study (Marancik et al., 2015) showed that R-line fish had higher igm (the total of secreted and membrane heavy chain mu: secHCmu and memHCmu) mRNA transcript levels in R-line compared to S-line 5 days post *Fp*-challenge. Langevin et al did not detect any significant changes in igm and igt gene expression in anterior kidney of naïve or experimentally challenged RBT in clonal lines of resistant and susceptible rainbow trout (Langevin et al., 2012).

Several studies have shown that there is increased accumulation of IgT^+ B cells in mucosal tissues after an experimental challenge with a pathogen (Olsen et al., 2011; Salinas et al., 2011; Tongsri et al., 2020; Zhang et al., 2010). In a review by Salinas et al, the immune response in gut-associated lymphoid tissue (GALT) to parasites was associated with IgT^+ B cells (Salinas et al., 2011). Using immersion vaccination with a polyvalent inactivated *Fp* vaccine, an increase in IgT^+ B-cells was detected in systemic organs, HCtau mRNA transcript in hindgut and total IgT in serum through ELISA (Hoare

et al., 2017). Olsen et al also showed that immunizing fish by intraperitoneal infection of live theronts (a life stage of a pathogenic ciliate) increases membrane HCtau (memHCtau) mRNA transcript levels in the gills. This upregulation was interpreted to be protective after challenging the immunized fish again with live theronts (Olsen et al., 2011). Altogether, these studies suggest that oral and immersion routes increase IgT levels in one form or another in mucosal tissues, whereas intraperitoneal or intramuscular routes increase IgM in systemic organs (Castro et al., 2013; Hoare et al., 2017; Makesh et al., 2015; Tongsri et al., 2020). In agreement to this, Castro et al reported both IgM^+ and IgT^+ B-cell populations in spleen; IgM^+ B cells being more clonally diverse and complex implying that IgT^+ B-cells might not get activated there (Castro et al., 2013). Depletion of IgT^+ B cells significantly increased the susceptibility of juvenile rainbow trout to *Ichthyophthirius multifiliis* infection and disrupted the gill microbiome (Xu et al., 2016). These results suggest that IgT secreting cells either travel to the mucosal sites after activation in spleen, or that after development, move to reside in the mucosal tissues and are activated locally. However, further studies are required to validate this hypothesis.

Corticosteroids are critical in regulating the metabolic as well as immune pathways in fish, primarily through cortisol. One aspect that has not been studied extensively is how stress influences the resistance of RBT against *Fp*, more specifically: how cortisol levels affect the anti-bacterial humoral immune response. Stimuli like crowding, handling, moving and disease lead to stress responses in hatcheries and aquaculture-raised salmonids (Yarahmadi et al., 2016), while migrating *Oncorhynchus* species have highly elevated levels of cortisol (Baker and Vynne, 2014; Carruth et al., 2000). Cortisol is known to induce suppression of several key mechanisms of the teleost humoral immune system, including B cell development, activation and proliferation (Tripp

et al., 1987; Verburg-van kemenade et al., 1999). When carp immune tissue cultures were treated with cortisol, this resulted in both induction of apoptosis and reduced B cell proliferation in peripheral blood (PBLs), anterior kidney and spleen (Verburg-van kemenade et al., 1999). Another *in vivo* study in common carp showed cortisol-induced apoptosis specifically in Ig+ activated B cells (Weyts et al., 1998). Similarly Espelid et al (Espelid et al., 1996) showed that *in vivo*, the number of Ig+ B cells in peripheral blood dramatically reduced after a cortisol injection in Atlantic salmon. A slow release cortisol implant study in RBT showed strong modulation of gene expression of different components of innate immunity including the complement system(Cortés et al., 2013), but effects on HCmu or HCtau expression were not studied. Post challenge with parasites, Olsen et al reported that hydrocortisone treatment significantly decreased the HCtau expression in gills whereas HCmu was significantly increased in both non-hydrocortisone treated and mock groups (Olsen et al., 2011).

Humoral immune activation substantially increases gene expression of the secreted form of Igs, as compared to its membrane forms, in secondary immune organs such as the spleen. A change in secreted to membrane Ig ratio has been shown to be an effective measure of B cell activation in other studies (Langevin et al., 2012; Zwollo et al., 2017). Here, we investigated the effects of cortisol and *Fp*-challenge on B cell activation. Specifically, we hypothesized that chronically elevated cortisol levels suppress the Ig response, and correlate with increased pathogen loads in fish. We tested this in the spleen by measuring pathogen load and changes in the ratio of secHC to memHC mRNA, for both mu and tau genes, using RT-qPCR. Additionally, we measured possible effects on B cell development by measuring changes in gene expression of memHCmu and memHCtau in anterior kidney. Lastly, possible effects on

the abundance of IgM^+ and IgT^+ Ig-secreting cells (ISCs) in blood were measured by flow cytometric analysis of purified PBLs.

Our results show that cortisol implants delay splenic IgM activation in both lines after *Fp*-challenge. Further, the IgM response in *Fp*-challenged fish with cortisol implants is exacerbated in S-line fish but not in R-line fish, while their pathogen loads are also higher. Overall, our data suggests that the IgM response, prevalence of *Fp* and severity of infection in *Fp*-resistant RBT is less sensitive to increased cortisol levels as compared to *Fp*-susceptible fish.

METHODS

Animals and facilities. Year-class 2018 ARS-*Fp*-R and ARS-*Fp*-S-lines of RBT (Silverstein et al., 2009; Leeds et al., 2010) were bred and reared at the National Center for Cool and Cold Water Aquaculture(NCCWA) following NCCCWA Institutional Animal Care and Use Committee procedures. The two lines were created by differential selection applied to the same base population. The ARS-*Fp*-R line was selected for 5 generations for increased survival while the ARS-*Fp*-S line was selected for one generation for decreased survival and subsequently randomly bred within line. Prior to the start of this experiment, RBT were transported to the animal facility at William & Mary and acclimatized to lab conditions in 100-gallon tanks for 2 weeks. Water temperature in the tanks was maintained at 12°C and recirculated through biological filters. A 12 hour-light/12 hour-dark cycle was maintained throughout the experiment. This study was approved by the W&M IACUC committee (IACUC-2018-11-26-13267-pxzwol). All the experiments were designed to minimize the pain and suffering for all animals used in this study.

Experimental design and Sampling procedure. The average body weight of RBT was 193.2 ± 44 g for R-line and 229.7 ± 52.9 g for S-line fish. Before treatment, trout were transferred to a dedicated challenge room to prevent pathogen transmission. RBT lines were kept in separate water tanks in the challenge room, where they acclimatized for 4 days. On day -5, the trout were anaesthetized with 0.1 g/L MS-222 (Tricaine-S, Western Chemical, Inc.) and intraperitoneally injected either with autoclaved coconut oil (virgin, certified organic, Sigma-Aldrich) mixed with 50 ug cortisol (Hydrocortisone 21-hemisuccinate, Sigma-Aldrich) per fish (treatment) or coconut oil alone (mock). Cortés et al. (Cortés et al., 2013) shows that after cortisol implants, serum cortisol levels were elevated on days 1 and 5, and return to basal levels on day 10. We opted to infect trout after 5 days of implant to emulate chronic stress conditions (day -5; Figure 1B). On day 0, trout were anesthetized as before and intramuscularly injected below the cranial aspect of the dorsal fin with either *Fp* culture (4.4E7 cfu/fish; 0.1mL of freshly grown *Fp* CSF259-93 in sterile PBS) or mock (0.1 mL sterile PBS). The number of viable *Fp* colony forming units were determined by plate count. Each line had 4 treatment groups for a total of 8 groups, as shown in Figure 1A. A representative timeline of a cortisol implant followed by *Fp*-challenge is shown in Figure 1B. Fish handling was kept to the minimum required time and fish were immediately returned to their tanks after injections. RBT were observed until they regained proper swimming. Some of the fish in all cortisol treated (C+) groups exhibited dorsal swelling just posterior to the skull (see Supplemental Figure 1; (Kent et al., 1989)). Morphological changes due to stress response in fish have been reviewed in Harper and Wolf (Harper and Wolf, 2009). S-line fish treated with both cortisol and *Fp* (C+*Fp*+) showed the most severe symptoms as compared to the other treatment groups, including spleen and liver necrosis, dark black spots on the liver, and lesions around the tail. Behaviorally, S-line fish in the C+*Fp*+

group displayed both erratic swimming patterns and lethargic behavior, while (C+*Fp*+) R-line fish only displayed lethargic behavior.

After *Fp* challenge, 3-5 trout were randomly sampled on Day 1, Day 3 and Day 5 and euthanized with 1 g/L MS-222 in PBS. Trout were bled immediately; blood was kept overnight at 4°C and sera separated by centrifugation and stored at -80°C for future use. The spleen index [spleen weight (mg) / body weight (g)] was determined at the time of dissection and recorded for each fish. Spleen index did not differ across lines ($F=0.37$, $p=0.54$), nor was it different between treatment groups ($F=0.12$, $p=0.94$) or days post infection ($F=0.02$, $p=0.97$). Further, no significant interactions were reported in our ANOVA model, meaning that the spleen index did not differ when it was compared across individual days across lines and treatment groups. This result was not unexpected because of low N values (3 or 4) per treatment group. (Data not shown).

Approximately 0.1 g of immune tissue (spleen and anterior kidney) were stored in RNAlater (Invitrogen) in -80°C for future nucleic acid purification.

Nucleic Acid Purification. Genomic DNA was purified from spleen tissue using DNAzol, according to the manufacturer's instructions (Molecular Research Center, Inc.) using ~20 mg of tissue and 1 mL DNAzol, as described previously (Chappell et al, 2017). RNA was extracted from ~20 mg of spleen or anterior kidney samples stored in RNAlater as described previously (Schouten et al 2013). Purified DNA and RNA was stored at -80°C until further use. cDNA was generated from RNA using iScript™ Reverse Transcriptase Supermix for RT-qPCR (Bio-rad), according to manufacturer's directions.

Real-time RT-PCR. RT-qPCR was performed to quantify gene expression of memHCmu and secHCmu using primers and reagents as previously described (Schouten et al., 2013). A Taqman assay was used to determine gene expression of

secHCtau and memHCtau, as described previously (Zwollo et al. 2017). Ratio of secreted Ig over membrane Ig was calculated as a measure of Ig response for both mu and tau in spleen (from here on referred to as "MU ratio" and "TAU ratio" respectively). For anterior kidney, a third set of primers was used to amplify α-tubulin (Schouten et al, 2013); the 2^−Δ(ΔCT) method as described by Livak and Schmittgen, 2001 was used to determine levels of memHCmu and memHCtau relative to the α-tubulin endogenous control. Quantification of *Fp* load by copy number was also done using RT-qPCR from splenic gDNA, as described previously (Chappell et al, 2017).

Purification of PBLs and Flow cytometry. PBLs were purified from blood using Histopaque 1077 (Sigma Aldrich) as described previously (Zwollo et al, 2005). Cell density of purified PBLs (number of cells/ml) was determined using a hemacytometer. Abundance of IgM^{++}/IgT^{-} and IgT^{++}/IgM^{-} Ig-secreting cells (ISCs), as well as IgM^{+} (IgM^{+}/IgT^{-}) and IgT^{+} (IgM^{-}/IgT^{+}) B cells was determined by flow cytometry as described previously (Zwollo et al., 2017).

Competitive Cortisol ELISA. The ELISA protocol was from (Barry et al., 1993) and a cortisol extraction protocol from Dr. Cory Champagne (personal communication) with modifications: Serum samples were thawed completely and vortexed for 5 seconds prior to use. Cortisol extraction was performed using diethyl ether (HPLC grade, Fisher Sci.). 100 uL of serum was mixed vigorously by vortexing for 60 seconds in 1 mL of diethyl ether. The mixture was then allowed to separate for 10 minutes at room temperature. Complete phase separation was achieved by centrifuging the samples for 5 minutes at 3000 rpm. Separation was followed by freezing the aqueous layer at -80°C for 10 min. Next, the supernatant was separated from the aqueous layer by pouring it in a new 1.5 mL epp. After a second extraction using 500 uL of diethyl ether, the supernatants were combined and evaporated in a SpeedVac Concentrator (Thermo Scientific) at RT for 30

minutes. The extract (containing cortisol) was dissolved in 100 uL Assay buffer [0.04 M sodium phosphate (monobasic, monohydrate), 0.06M sodium phosphate (dibasic, anhydrous), 0.87% NaCl] by vortexing the samples for 30 min and stored at -20°C until further use. Exogenous (control) cortisol samples for the standard curve were prepared by resuspending Hydrocortisone 21-hemisuccinate (Sigma-Aldrich) in 100 uL of charcoal-stripped serum to make a stock of 102.4 ng/mL. This exogenous cortisol standard was then extracted using 1 mL diethyl ether and extracts resuspended in 100 uL Assay buffer without any bovine serum albumin (BSA). This extracted exogenous cortisol standard was then diluted 4-fold to make 7 standards in the range of 0.25 to 102.4 ng/mL. ELISA plates (96 well Flat-bottom Immuno plate, MaxiSorp, Thermoscientific) were coated with 50 uL of anti-cortisol antibody to a final dilution of 10ug/mL (Invitrogen, cat # PA1-85346) in coating buffer [0.035M Sodium Bicarbonate, 0.015M Sodium Carbonate] and incubated at 4°C for 16 hours. Plates were washed 5 times with wash solution [Tween 20 (0.05%), 0.15M NaCl] (Barry et al.). Both the extracted exogenous cortisol standards and endogenous cortisol (serum) samples were diluted 1:64 in Assay buffer prior to use. 0.1% BSA was added to assay buffer on the day of the assay. Cortisol-HRP conjugate solution (CHRPS) was prepared by dissolving 16 uL of Cortisol-HRP conjugate (CHRP) (Fitzgerald Industries International) in 7996 uL (1:500 dilution) of freshly prepared assay buffer with BSA for ~48 samples. 150 uL of CHRPS was added to all samples and standards (total final volume was kept at 300uL), for a final dilution of 1:1000 of CHRP. 100 uL of each standard and sample was added to designated wells. 100 uL of CHRPS only was added in maximum binding wells (MAX) and non-specific binding (NSB) wells. The plate was incubated at RT on a rotating platform for 2 hours. Next, the plate was washed 5 times in washing solution and 100 uL freshly prepared OPD substrate (Thermofisher) solution added. Absorbances were read using an ELISA microplate reader (BioRad iMark) at 490nm after 25 minutes. Percent

Max binding Assay was used to generate standard curve and concentrations of cortisol in the serum samples using % CHRP bound and comparing it to MAX binding wells (B/Bo).

Statistical analysis

The explanatory variables used in ANOVA model were line (L), treatment groups (TG) and days post-infection (DPI). The levels of each variable were: L (R, S), TG (C–/*Fp*–, C–/*Fp*+, C+/*Fp*–, C+/*Fp*+), and DPI (1, 3, and 5). Potential physiological changes, including spleen index, pathogen load, and serum cortisol levels, were assessed. A 4 (TG) x 3 (DPI) x 2 (L) ANOVA was calculated individually for the three response variables, as described below. Tukey post hoc analysis was done following ANOVA's. Bodyweight of fish did not significantly affect the fish cortisol levels or pathogen load, as confirmed by using body weight as a covariate in ANOVA analysis (data not shown).

Data Transformation. Pathogen load, PBL cell density, MU and TAU ratio (spleen), and HCmu and HCtau (anterior kidney) values were log-transformed to meet the assumption of normality and homogeneity of variance for ANOVA. Integer 1 was added to the pathogen load data before log transformation to avoid infinite values on the log scale for 0 values of pathogen load.

Analysis of variance tests. Multiple three-way type II factorial ANOVAs' were carried out separately to analyze the response variables: MU and TAU ratio in spleen, Fold change for memHCmu and memHCtau in anterior kidney, serum cortisol levels, spleen index, cell density of PBLs, and splenic pathogen load. Tukeys posthoc tests were performed after ANOVA's to check for significant differences ($p \leq 0.05$ as threshold, $p \leq 0.1$ are also reported) among days in respective lines and treatment

groups. The Tukey posthoc test reports adjusted p-values which account and correct for false type I errors.

Strip Charts. All strip charts were made using ggplot2 package in R environment. Average values are shown in solid circles and individual data points are shown by open circles. Error bars are standard errors.

Linear modeling. To check for multiple correlations, a linear model was made using the MU ratio (log-transformed) as a dependent variable, and serum cortisol levels and pathogen load as independent variables. Both independent variables were scaled to average values (each data point was subtracted from the average value) to meet requirements of normality and homogeneity of variance in data. 'Effects' package in R was used to extract predicted values from the linear model and were plotted using typical values of independent variables (0-5 for Pathogen load; Mean ± 2 SD for serum cortisol levels).

RESULTS

This study sought to understand the effects of cortisol implants on Ig response and clearance of *Fp* infections in RBT. Fish were intraperitoneally injected with slow-release cortisol implants (C+) followed by an experimental challenge with *Fp* (*Fp*+) in two separate lines of RBT: a genetically *Fp*-resistant (R) and a *Fp*-susceptible (S) control line. Ig response was measured as the ratio of secreted to membrane forms of HC, for the two major Ig classes in RBT, IgM and IgT. Further, it was determined whether the Ig response after cortisol implants was protective or not, as implied by pathogen load.

Changes in pathogen load between genetic lines and effects of cortisol implant

We measured splenic *Fp* load in each fish to investigate differences between lines and to examine the effect of implanting cortisol. Pathogen load was measured on

Days 1, 3, and 5 Post Infection (DPI) (Table I). As expected, sham-challenged fish exhibited no detectable *Fp* load and only *Fp*-challenged data were included in the ANOVA analysis. The ANOVA analysis showed significant main effects of pathogen load for all three independent variables: TG ($F=4.62$, $p=0.04$), DPI ($F=12.7$, $p < 0.001$) and L ($F=33.2$, $p <0.001$)]: S-line, TG C+*Fp*+, and Day 3 and 5 had a higher pathogen load (Table IIA). In addition, there were significant two-way DPI x L interactions ($F=4.2$, $p=0.02$) and significant three-way TG x DPI x L interactions ($F=5.06$, $p=0.01$).

In the mock implant groups (C–*Fp*+), S-line fish had a higher *Fp*-prevalence than R-line fish (66% on Days 1 and 5 and 100% on Day 3 for S-line, 33% on all three days for R-line; Table IA and IB). Furthermore, S-line fish also had a significantly higher *Fp* pathogen load compared to R-line, but only on Day 3 (S-line: M=42007*, R-line: M=4.76*; Table II, Figure 1A; M is the mean copy number of *Fp* as measured by RT-qPCR). Lastly, the pathogen load declined by Day 5 for S-line fish, while R-line remained low on all three days (Figure 1A). These data are in agreement with earlier studies (Marancik et al., 2015; Zwollo et al., 2017).

The *Fp* load was significantly higher in the cortisol implant groups as compared to mock implant groups (Table II; Compare G3 and G4 under Treatment Groups). In S-line fish, the *Fp* load in fish with cortisol implants was significantly higher compared to fish with mock implants, but only on day 5 (compare Figure 2A and 2B). In R-line fish, *Fp*-loads did not differ considerably between mock (C–*Fp*+) and cortisol implanted groups (C+*Fp*+) on different days as indicated by post-hoc analysis. Importantly, in R-line fish, *Fp* loads dropped sharply between Day 3 and Day 5 (from M=1197 to M=21) but not in S-line fish (from M=49637 to M=22141; Figure 2B). For S-line fish, M values remained significantly higher than on Day 5 compared to Day 1 (Figure 2B). In summary, these data suggest that R-line fish sustained a low pathogen load with no significant

effect of cortisol treatment by Day 5 as compared to high pathogen load in S-line (compare Figure 2A and 2B, Day 5).

Lastly, Fp-challenged R-line fish with cortisol implants (C+*Fp*+) had a higher *Fp* prevalence compared to R-line fish with mock implants (C–*Fp*+) (100% and 33% respectively on Day 3; data not shown). In contrast, cortisol-treated (C+*Fp*+) S-line fish had 100% prevalence on Day 3, the same pattern as in mock implanted (C–*Fp*+) S-line fish (Table IA and IB).

Changes in serum cortisol levels due to implant and Fp challenge

To determine how cortisol implants and *Fp* challenge affected endogenous serum cortisol levels in fish, we used a competitive ELISA. ANOVA results listed in Table IIB indicated that there were significant main effects for L ($F = 12.83$, $p < 0.001$) and TG ($F = 12.55$, $p < 0.001$) towards endogenous serum cortisol levels. Additionally, there were marginally significant DPI x L and TG x DPI interactions towards serum cortisol levels. Interestingly, based on the ANOVA data, the cortisol levels in S-line were higher than in R-line fish, both for Day 3 and Day 5. In addition, C+*Fp*+ fish had higher cortisol levels on Day 3 when compared to mock treatment groups. Other interactions were not significant. Tukey post hoc analysis revealed that average serum cortisol levels did not differ between days when grouped by both line and treatment ($p>0.05$). (Table IIB; raw data not shown).

Altogether, our ANOVA results suggest that R-line generally had lower serum cortisol levels than S-line and that the effect of the cortisol implant, alone, was not measurable during the study period. Furthermore, cortisol levels were elevated by *Fp* challenge. The non-significant differences, in majority can be attributed to low sample size in our study,

however, we strongly emphasize that the trend we report (Suppl. Figure 2) has a biological significance.

Changes in MU Ratio in the Spleen are affected by Fp challenge

Next, to investigate whether Ig gene expression was affected in our treatment groups, we used MU ratio as a proxy for B cell activation in the spleen. Using ANOVA, only TG had significant main effects ($F=29.2$, $p < 0.0001$); overall, *Fp* challenged groups had a higher MU ratio as compared to mock challenged groups, as expected (Table IIC). All the two way (Table IIC) and three-way interactions (Figure 3) reported in the model were highly significant; TG x DPI ($F=5.4$, $p<0.0001$), TG x L ($F=17.77$, $p<0.0001$), DPI x L ($F=28.5$, $p<0.0001$) and TG x DPI x L ($F=12.3$, $p<0.0001$).

In mock-challenged fish, cortisol implants did not significantly downregulate the MU ratio, independent of line [compare Figure 3A (no cortisol) and 3C (with cortisol)]. However, mock-implanted *Fp*-challenged fish (C–*Fp*+; Figure 3B) differed significantly between lines, including an *earlier* upregulation of MU ratio in R-line fish compared to S-line fish: in R-line fish, there was already an upregulation on Day 1, while in S-line, there was no upregulation until Day 3. Additionally, on Day 3, both MU ratio as well as *Fp* load were significantly downregulated in R-line fish, but not S-line fish (Figure 3B). S-line fish maintained a consistent upregulation of the MU ratio through Day 5 while pathogen load had dropped by Day 5 (Figure 3B).

This delayed MU response and pathogen clearance in S-line fish was also observed in the cortisol implanted *Fp* challenged (C+*Fp*+) fish (Figure 3D). However, in the presence of cortisol, the upregulation of MU ratio was delayed by another two days, and in both lines; in S-line fish, it was delayed from Day 3 to Day 5, and R-line delayed from Day 1 to Day 3 (Compare Figures 3B and 3D).

Interestingly, the cortisol-implant induced delay in upregulation of MU ratio had much milder effects on pathogen loads in R-line fish compared to the S-line fish (Compare Figure 3B and 3D): although the delay in response resulted in a somewhat higher pathogen load on Day 3, both the MU ratio and the pathogen load had declined by Day 5, pointing towards a protective Ig response in R-line fish (Figure 3D).

In summary, we report a delayed IgM response (delayed MU ratio increase) in the spleens of both *Fp*-challenged R-line and S-line fish that were treated with cortisol. Further, cortisol implants appeared to affect pathogen clearance in *Fp*-susceptible fish to a greater extend compared to *Fp*-resistant fish.

Changes in TAU ratio in the spleen

There were no significant differences in the splenic TAU ratio across TGs ($F=1.8$, $p=0.15$) or days ($F=0.42$, $p=0.65$). However, there were main effects of Line towards TAU ratio; the TAU ratio in R-line (M= -1.6) was generally lower as compared to S-line (M= -1.2) ($F=21.6$, $p<0.0001$), with relatively small fold changes in the TAU ratios (0.001-0.32 fold change) compared to the MU ratios (0.1-86.4 fold change). No other significant interactions were found (data not shown). In summary, this suggests very low activation responses detectable for IgT in the spleen, with R-line fish having less IgT activation than S-line fish. Hence, the splenic TAU ratio did not provide a useful approach to measure effects of cortisol on *Fp*-challenge in our study.

Changes in HC mu and tau expression in the Anterior Kidney

Anterior kidney in fish is analogous to bone marrow in mammals; it is the site where B cells develop. mRNA transcript levels of HCmu and HCtau, for both membrane and secreted forms, were determined to investigate changes in B lymphopoiesis. Expression was normalized using the α-tubulin gene. Table IIIA shows results from

ANOVA analysis; means and standard errors are reported in Table IIIB. For memHCtau and secHCtau, there were no main or interaction effects reported in the ANOVA analysis. For memHCmu and secHCmu, there were significant main effects of line (memHCmu: $F=5.04$, $p=0.03$, secHCmu: $F=9.80$, $p<0.001$); S-line was generally higher than R-line, in agreement with an earlier study (Zwollo et al, 2014). Other significant effects are reported in Table III; however, a conclusive trend could not be established. Hence, measuring gene expression of memHCmu or HCtau in the anterior kidney did not provide useful information to measure effects of cortisol on *Fp*-challenge in our study.

Effects on cell density and abundance of PBLs

Striking differences in PBL cell density were observed between S and R-line fish. In R-line fish, cortisol implants and/or *Fp*-challenge did not affect PBL density for any time point (Suppl Fig. 3). For S-line, in the absence of cortisol implants, *Fp*-challenge significantly reduced PBL densities, but only on day 3 post infection; PBL densities were recovered by day 5, with values similar to the control groups and R line fish. Importantly, in cortisol treated *Fp*-challenged S-line fish, PBL density values dropped on day 3 but remained significantly lower on day 5 compared to fish with no cortisol implants on the same day (suppl. Figure 3). Hence, *Fp*-challenged S-line fish had a sustained reduction in PBL density in the presence of cortisol implants, supporting a continued cell migration response to *Fp* in the presence of cortisol, with wbcs likely leaving the bloodstream to enter sites of infection (Espelid et al., 1996). (ANOVA data not shown)

Next, we also sought to investigate potential changes in the abundance of IgM^{++}/IgT^{-} and IgT^{++}/IgM^{-} Ig-secreting cells (ISCs), as well as IgM^{+} (IgM^{+}/IgT^{-}) and IgT^{+} (IgM^{-}/IgT^{+}) B cells in blood, using flow cytometric analysis of PBLs from fish from each

group, however no significant differences between the groups were found (Data not shown).

Assessing Correlation results

To further explore cause and effect and investigate if serum cortisol levels and pathogen loads were correlated in our study, a linear regression with multiple variables (Figure 4) was performed separately for each line. Figure 4 shows that in both lines, there was a positive correlation between serum cortisol levels and pathogen load. This correlation was stronger in the S-line. In addition, there was a strong negative correlation between PBL density and pathogen load, but only in S-line (Figure 4).

Correlation analysis indicated that the MU ratio in spleen positively correlated with both pathogen load and serum cortisol levels, with S-line having a stronger correlation ($R>0.5$) as compared to R-line ($R<0.5$) (Figure 4A and 4B). After performing linear regression on the independent variables, multiple linear regression was performed to explore the data further and look at the combined effect of pathogen load and serum cortisol. As indicated by summary tables of linear model (Table IVA and IVB), the interaction term Average_cortisol*Pathogen_load was significant in both lines ($p<0.05$). On the basis of multiple linear regression models, a line graph was plotted using the effects package in R (Figure 5A and 5B). Fitted values of the response variable (MU ratio) were plotted against typical values for explanatory variables (Serum cortisol: Mean value $\pm$ 1 SD, and pathogen load: 1-5). This was done separately for both lines.

According to the model, in S-line, for cortisol levels $\leq$ 69.9 ng/ul (one standard deviation above mean), the MU ratio increases from lower to higher as pathogen load increases. Mu ratio is a linear function of pathogen load. However, when cortisol levels cross a certain threshold, the MU ratio persistently remains high irrespective of pathogen

load, suggesting a more robust but presumably ineffective IgM response. In contrast, in R-line, the ratio increases with increasing pathogen load but only at low cortisol levels (17 ng/ul; one standard deviation below mean). At ≥43.47 ng/ul cortisol, the MU ratio remains unaffected and decreases when pathogen loads ≥ than 69.9, suggesting a reduced IgM response with increased pathogen loads under high cortisol conditions in R-line fish (Figure 5A and 5B).

DISCUSSION

Stress and increased cortisol levels have long been known to exacerbate disease expression in both aquaculture and wild salmonid populations. While several studies have shown selective breeding to be a successful mechanism to combat pathogenic outbreaks, the effects of cortisol on disease resistance remain poorly understood. Here, we examined the effects of cortisol implants followed by *Fp*-challenge in a *Fp*-resistant and *Fp*-susceptible line of RBT with the aim to see how Ig response was affected. Comparing *Fp*-challenged fish only, we report an earlier and effective IgM response in R-line as indicated by lower pathogen loads, when compared to S-line fish. Moreover, in *Fp*-challenged fish with cortisol implants, there is an upregulation of endogenous cortisol in both lines, although higher in S-line than R-line. Interestingly, while R-line fish could remove *Fp* efficiently by Day 5 post-infection, S-line fish could not. Further, the splenic IgM response, measured as ratio of secreted to membrane form of heavy chain mu transcripts (MU ratio), was delayed in cortisol implanted fish in both lines.

Both the MU or TAU ratio were used as a proxy for immune stimulation and activation in our study, but this ratio is a relative measure. Biologically, an increased ratio could mean either an increase in secreted form of the heavy chain, or a decrease in the membrane form. If both secreted and membrane forms increase or decrease at a

comparable pace, any actual change would not be detected. However, that is highly unlikely in the spleen because ISCs produce up to 100 fold higher levels of the secreted forms of the HC transcripts after immune activation, compared to levels of membrane HC transcripts in resting B cells. Hence, these ratios, as a proxy for immune response have been used by us and others as a reliable approach to measure B cell activation (Peterson, 2007; Zwollo et al., 2017).

Exacerbated IgM response in cortisol-treated S-line fish.

The quantification of mRNA transcript levels, cortisol levels and pathogen load over time in both lines proved to be useful in evaluating immune response in selectively bred RBT, particularly in the spleen. S-line fish with cortisol implants had an *exacerbated* IgM response as indicated by the high MU ratio. We speculate that this IgM response was not protective, as confirmed by high pathogen loads in these fish. Along with higher pathogen load, S-line also showed more necrosis in liver and spleen and more behavioral changes as compared to R-line fish, which suggests the immune response had a detrimental role towards health of fish. One caveat to this interpretation is more internal damage could be due to higher pathogen load and *Fp* multiplying in other organs which was not measured in this study. From linear modeling, we extrapolate that supra-physiological levels of serum cortisol result in an exacerbated immune response and a vicious circle of increased pathogen load and increased immune response. It is important to note that pathogen load was measured only in the spleen, other organs such as anterior kidney, muscles and site of infection might also harbor the bacterial pathogen which was not measured. Hence, our assumption here is offered with precaution.

Importantly, the *Fp*-challenged R-line fish were less compromised as compared to S-line fish, with and without cortisol: their pathogen load was lower, their IgM

response (MU ratio) was much milder, and no significant reduction in PBLs was observed. This leads us to propose that R-line fish eliminate the pathogen with a mechanism other than Ig response and/or clears it out with a more effective (higher affinity) antibody response. It has been reported in humans that variable regions of Igs are involved in a more efficacious protective response (Bryson et al., 2016; Giuliani et al., 2018). Marancik et al. (Marancik et al., 2014) suggested that the histopathological evidence after *Fp*-challenge in R and S-line demonstrates that survival rate is a result of differential disease magnitude and not altered disease course between genetic lines. In summary, the differential MU ratio suggests that mechanisms of elimination of the bacterium differ between R and S-line fish, and which affects disease magnitude.

Potential roles and placement of IgT

An earlier study from our lab and several other studies have suggested that Ig class IgT contributes to resistance or protection to *Fp* in RBT (Hoare et al., 2017; Olsen et al., 2011; Zwollo et al., 2017). In our study, we did not observe significant changes in IgT transcripts in the anterior kidney, TAU ratio in the spleen, nor IgT^+ cell abundance in blood, in fish under any treatment conditions. This result is perhaps not unexpected, as IgT^+ cells are localized at mucosal tissues such as gills or GALT (Olsen et al., 2011; Salinas et al., 2011), which were beyond the scope of the present study. Future studies should include such immune sites to determine possible effect of cortisol on IgT responses to *Fp*. However, it appears that cortisol implants prior to *Fp*-challenge did not change the development of IgT producing B cells in the anterior kidney of either line, at least not under our experimental conditions.

Factors influencing results presented in this study

In the current study, the cause of upregulation of MU ratio in spleen is unclear. An important thing to keep in mind is that cortisol implant or *Fp*-challenge alone did not increase endogenous cortisol levels in R or S-line after day 9 post-implant injection. Elevated cortisol may have occurred earlier (prior to Day 1 post *Fp*-challenge) but this was not analyzed in our study. Interestingly, cortisol levels were generally lower in R-line as compared to S-line fish. Effects of bacterial infection on cortisol responses in fish is a poorly understood area of research, owing to the complicated endocrine-pathophysiology interactions during infections. In our study, we artificially implanted cortisol in the peritoneal cavity of fish, which should have lead to higher endogenous serum cortisol levels based on other studies (Cortez et al 2013). However, cortisol implants were followed by *Fp*-challenge in fish, hence, infection also might have contributed to a rise in endogenous cortisol levels, as observed in our C–*Fp*+ and C+*Fp*+ treatment groups. Bacterial infections, either natural or experimental, have been shown to raise the endogenous levels of cortisol in Red drum fish (Robertson et al., 1987), RBT(Ackerman and Iwama, 2001; Ackerman et al., 2000; Pickering and Pottinger, 1989) and European eel (Schulz et al., 2019). Therefore, in the C+*Fp*+ group, it was difficult to determine the cause of upregulated cortisol in S-line. Interestingly in this treatment group (C+*Fp*+), R line had lower cortisol levels as compared to S line. Hence, one could argue that the higher cortisol levels in S-line might be due to higher *Fp* load. To take this one step further, the exacerbated IgM response in S-line also might be due to higher *Fp* load. Further clarification is needed to resolve cause and effect. Hence, it is imperative that future studies compare both lines at a similar *Fp* load to address this caveat, and further, to measure cortisol at earlier timepoints and to increase the N value for all treatment groups. In short, it could be argued that the cortisol-induced modulation of HCmu genes is in response to higher pathogen loads.

Growth related traits such as body weight had been linked to resistance of different pathogens by an earlier study in Atlantic salmon (Yáñez et al., 2014). Due to experimental limitations and sample of convenience, by chance S-line fish had greater body weight as compared to R-line fish in our study. However, when body weight was analyzed as a co-variate in our ANOVA tests, it did not affect our response variables (data not shown). In addition, S-line fish despite higher body weight showed more pathogen load as compared to R-line; pointing towards the fact that this was a favorable bias because it eliminates the idea that body weight might contribute to greater or lesser pathogen load. In the current study, we did not find any differences between the lines with respect to spleen index and hence, spleen index was not further analyzed.

Future approaches and broader impacts

The basis of *Fp*-resistance in RBT is unknown at this time but may be associated with less inflammation, low but effective Ig response, rapid recovery of cortisol levels after stress and effective clearance of the pathogen through innate or non-specific immunity. Multiple studies have looked at changes in immune gene expression after *Fp*-challenge (Langevin et al., 2012; Marancik et al., 2015; Moore et al., 2019) or cortisol implant (Gadan et al., 2012; Philip et al., 2012; Cortés et al., 2013). In supplement to the humoral immune genes, these studies report changes in innate immune genes and signaling molecules, such as IL-10, complement factor c3, acute phase proteins SAA, Eosinophil-peroxidase-like and M-CSFR. To this end, it would be a fruitful approach to look at non-specific immunity in R-line fish. Furthermore, alternate approaches such as using a cortisol antagonist to block glucocorticoid receptors or dramatically increasing plasma cortisol levels through stressors to investigate its effect on disease progression in genetic lines might give us a good indicator about the basis of resistance. Once a similar disease progression in both genetic lines is achieved, factors such as specific

and non-specific immunity, phagocytic ability and pathogen clearance mechanisms can be explored further.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. (A) Experimental design showing 8 treatment groups challenged with mock/cortisol implants (C–/C+) and/or mock/*Fp* challenge (*Fp*–/*Fp*+) in Resistant (R) line and Susceptible (S) line of RBT. (B) A representative experimental timeline followed in treatment group C+*Fp*+, which shows the days for intraperitoneal cortisol implant followed by *Fp* challenge and tissue collection at different days post infection (DPI).

Figure 2. Strip charts showing pathogen load measured in spleen of mock cortisol and *Fp* challenged fish (A.) and cortisol implanted *Fp* challenged fish (B.). The Y-axis shows log transformed number of copies of *Fp* from RT q-PCR in 200 ngs of gDNA. The X-axis shows Days Post Infection. Individual data points (open circles), average values, and standard error bars for R-line are shown in blue and for S-line is shown in red. For number of copies of *Fp* as measured by q-PCR see Table I. Significant differences between days are shown in blue asterisks for R-line and red asterisks for S-line. Black

stars represent significant differences between lines on same days. Asterisks denote level of significance as follows: *, p<0.1; **, p<0.05; ***, p<0.001; ****, p<0.0001

Figure 3. Strip charts showing Ig response denoted by the ratio of secreted to membrane form of Heavy chain mu (MU ratio) on different days post challenge. The Y-axis shows log transformed fold change value of MU ratio in spleen. The X-axis shows Days Post Infection. Individual data points (open circles), average values and standard error bars for R-line are shown in blue and for S-line is shown in red. Size of open circles represents copy number of *Fp* as described in the figure legend. Values for MU ratio can be found in Table I. Significant differences between days are shown in blue asterisks for R-line and red asterisks for S-line, whereas black stars represent significant differences between lines on same days. Asterisks denote level of significance as follows: *, p<0.1; **, p<0.05; ***, p<0.001; ****, p<0.0001. **A.** Mock cortisol implants with mock *Fp* challenge; **B.** Mock cortisol implants with *Fp* challenge; **C.** Cortisol implants with *Fp* mock challenge; **D.** Cortisol implants with *Fp* challenge.

Figure 4. Visualization of correlation matrix of different variables for each line; R-line **(A)** and S-line **(B),** made in R. An absolute relationship is indicated by the coefficient value of 1, with +1 and -1 indicating an absolute positive or negative correlation, respectively. Correlation was calculated for five different continuous variables; Spleen index, serum cortisol levels, *Fp*-load, MU ratio, and TAU ratio. Dark red color indicates a stronger positive correlation and dark blue indicates a stronger negative correlation among the variables (Pairwise covariances and Pearson correlation coefficient were calculated by the ggcorr function in R).

Figure 5. Modelled probability of MU ratio as a function of pathogen load in Rainbow trout (*Oncorhynchus mykiss*) at three baseline cortisol (ng/mL) measurements: 17.04 = 1 SD below mean; 43.47 = Mean cortisol levels; 69.9 = 1 SD above mean; 96.33 = 2 SD

above mean; SD is standard deviation. Shaded area represents 95% confidence interval. Summary of linear models is shown in Table IVA and IVB for both lines, for R-line model, Adjusted R-squared: 0.1333; S-line model, Adjusted R-squared: 0.5533.

Suppl. Figure 1. Fish images showing observed symptoms in *Fp* challenged S-line fish with cortisol implants. Red arrows point toward the anomalies observed; a: Black spots on liver, b: Lesions around gills and mouth, c: Dorsal swelling

Suppl. Figure 2. Strip charts showing cortisol levels measured in the serum in ng/mL on different days post infection. The Y-axis shows cortisol levels in serum in ng/mL. The X-axis shows Days Post Infection. Individual data points (open circles), average values (solid circles) and standard error bars for R-line are shown in blue and for S-line is shown in red. Actual values for serum cortisol levels can be found in Table I. A. Mock cortisol implants with mock *Fp* challenge; B. Mock cortisol implants with *Fp* challenge; C. Cortisol implants with *Fp* mock challenge; D. Cortisol implants with *Fp* challenge.

Suppl. Figure 3. Bar charts showing log transformed cell density measured in the blood of peripheral lymphocytes on different days post infection. The Y-axis shows Cell density. The X-axis shows Treatment groups. Bar height represents mean values and standard error bars for R-line are shown in blue and for S-line is shown in red. Each facet in the grid represents days post infection. Significant differences between treatment groups are shown in blue asterisks for R-line and red asterisks for S-line, whereas black stars represent significant differences between lines within same treatment group. Asterisks denote level of significance as follows: *, $p<0.1$; **, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$.

Figures:

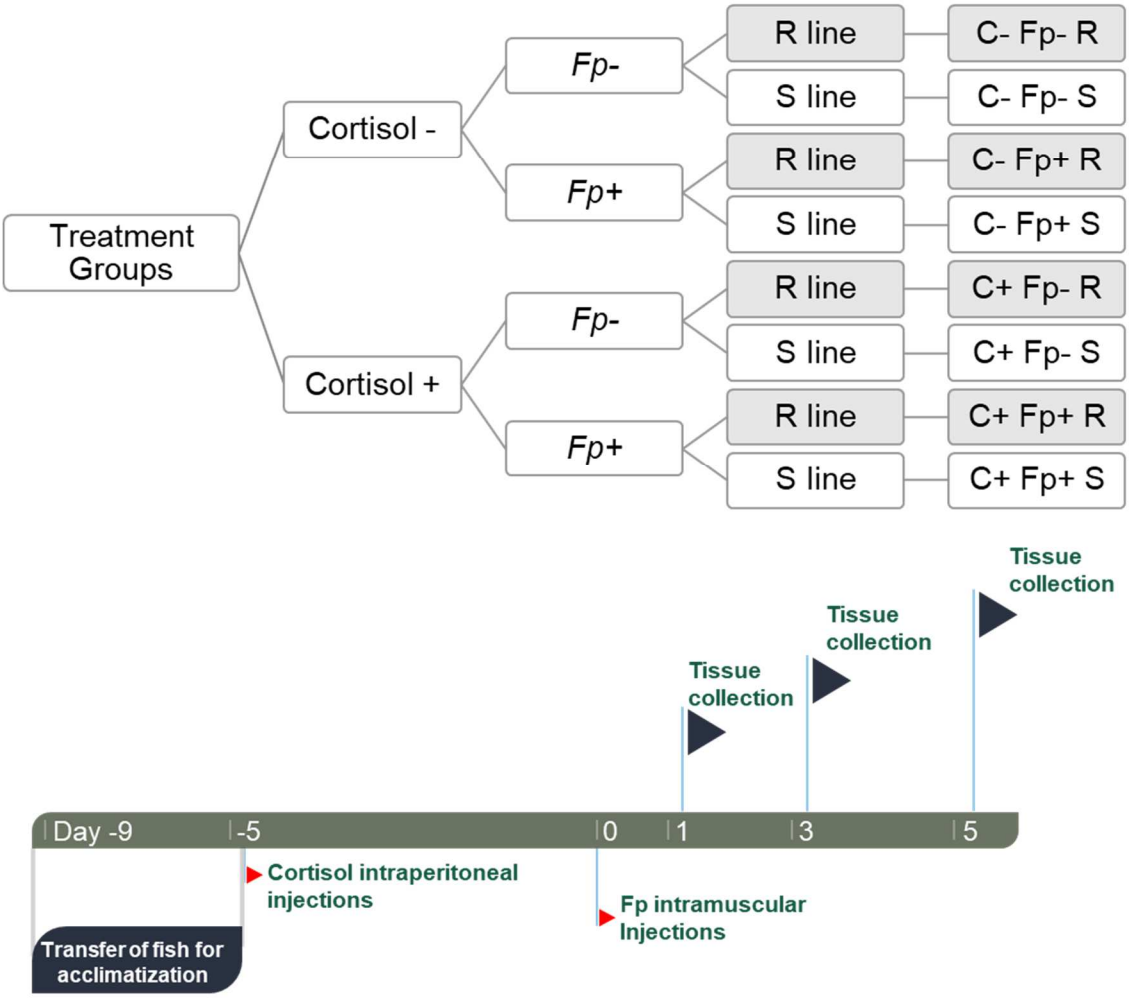


Figure 1

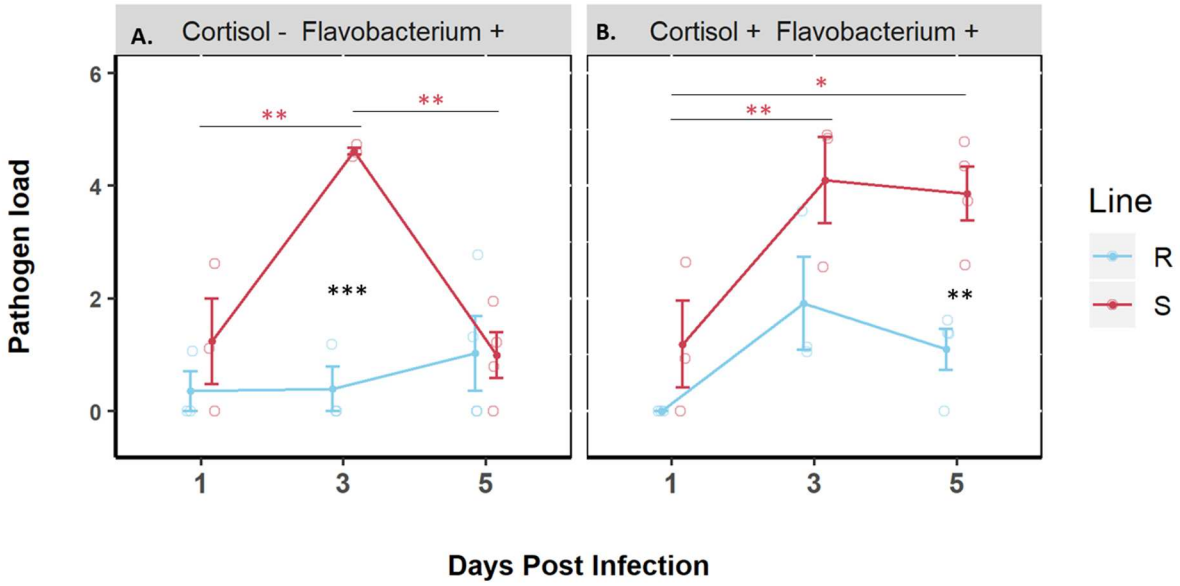


Figure 2

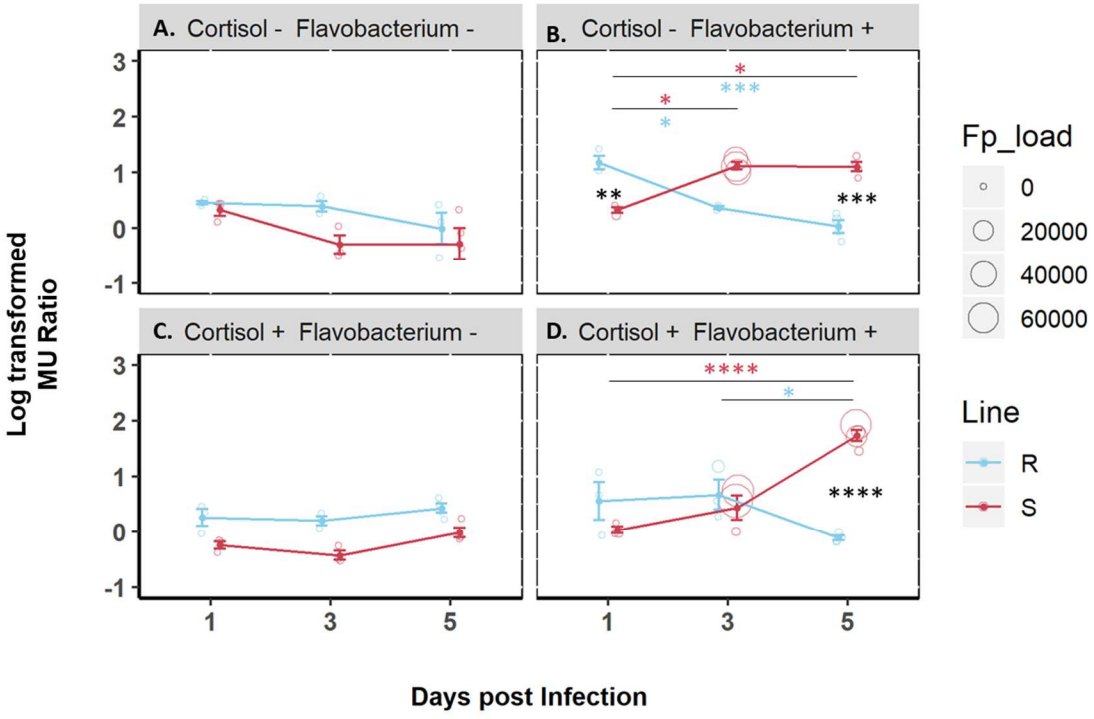


Figure 3

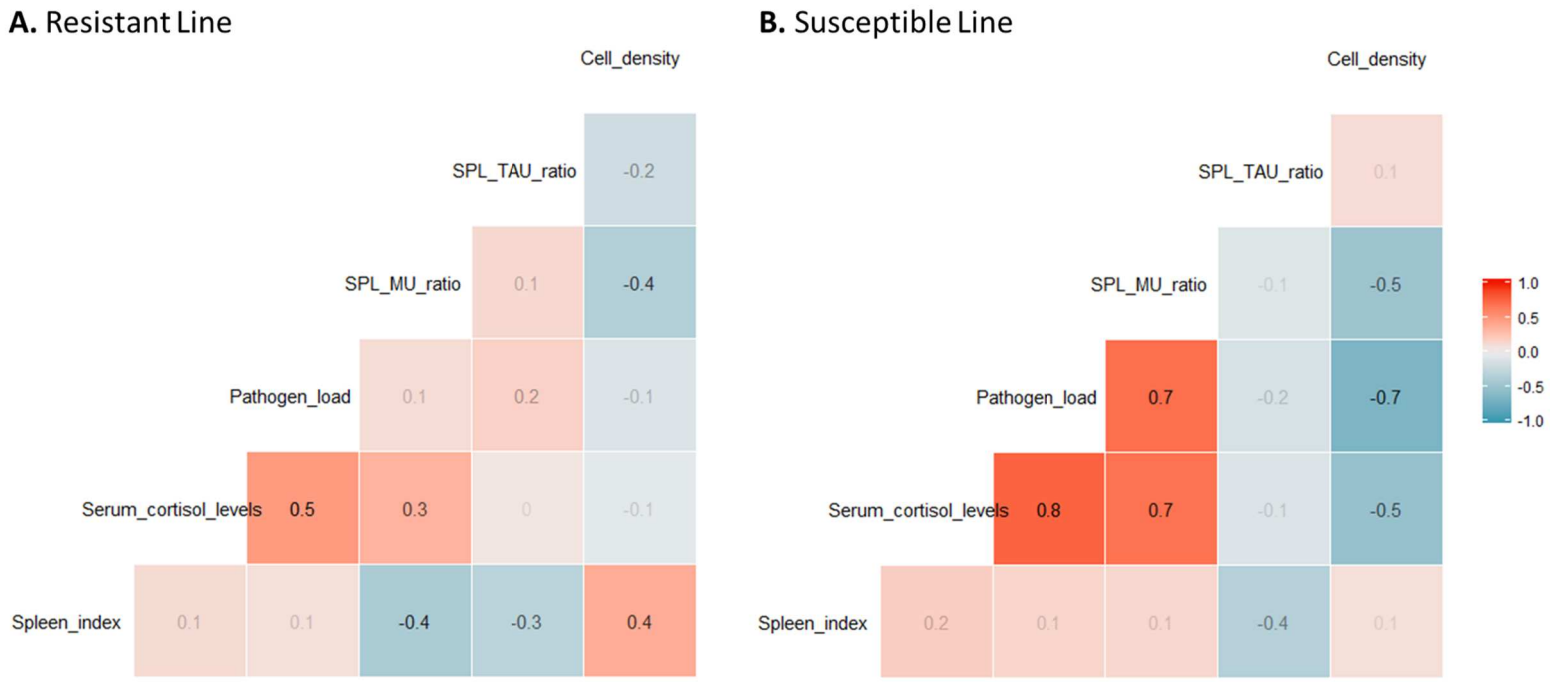

A. Resistant Line
Cell_density
SPL_TAU_ratio
SPL_MU_ratio
Pathogen_load
Serum_cortisol_levels
Spleen_index
-0.2
0.1
-0.4
0.1
0.2
-0.1
0.5
0.3
0
-0.1
0.1
0.1
-0.4
-0.3
0.4
B. Susceptible Line
Cell_density
SPL_TAU_ratio
SPL_MU_ratio
Pathogen_load
Serum_cortisol_levels
Spleen_index
0.1
-0.1
-0.5
0.7
-0.2
-0.7
0.8
0.7
-0.1
-0.5
0.2
0.1
0.1
-0.4
0.1
1.0
0.5
0.0
-0.5
-1.0

Figure 4

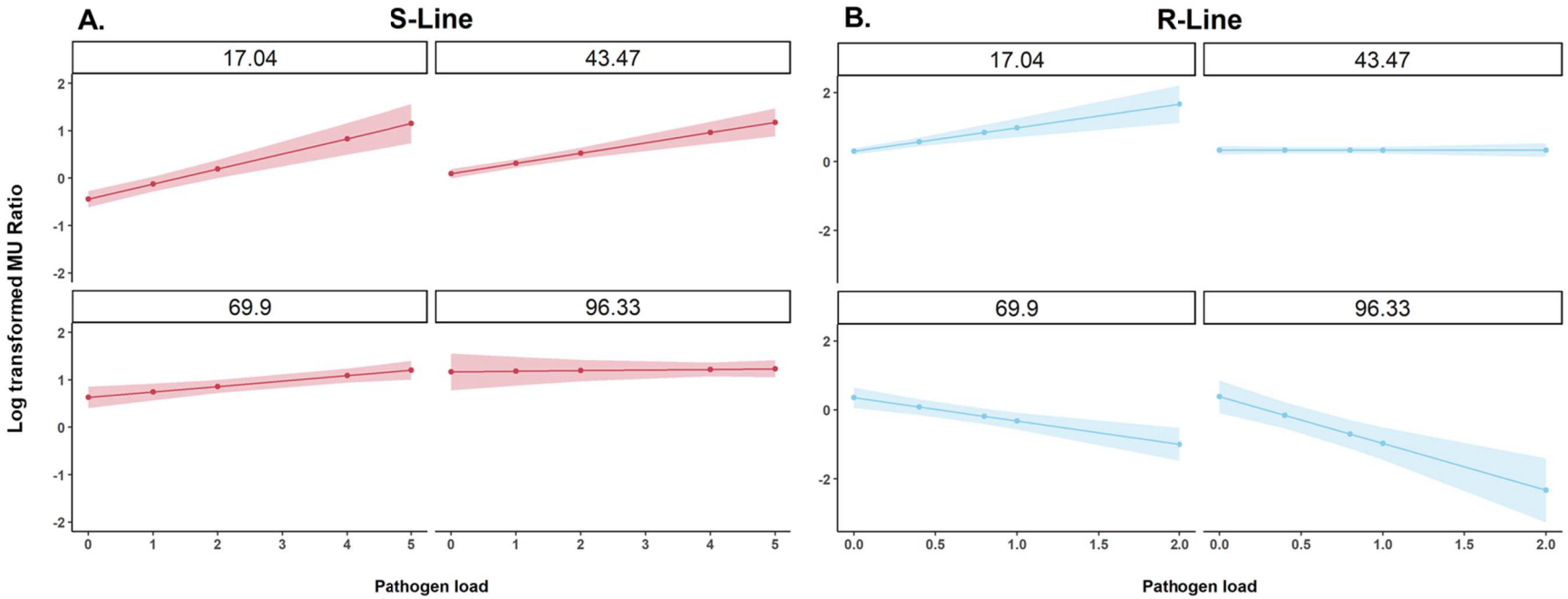
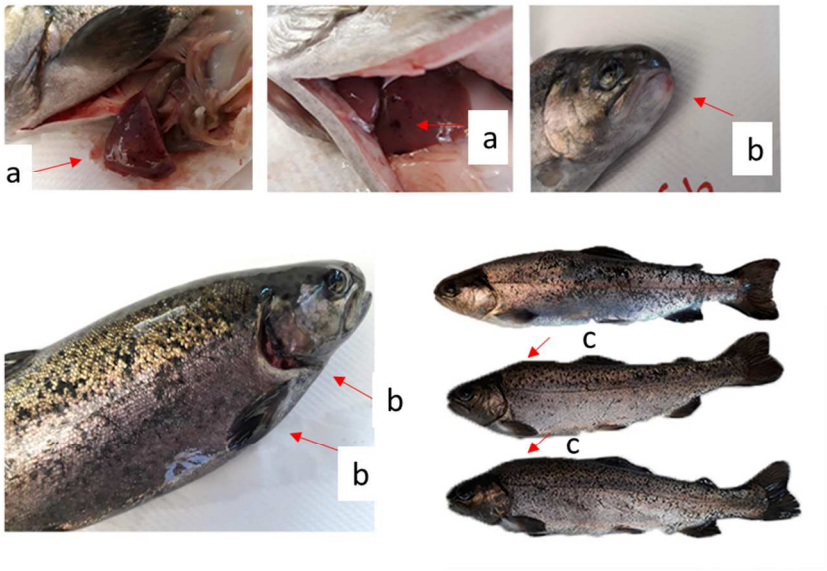
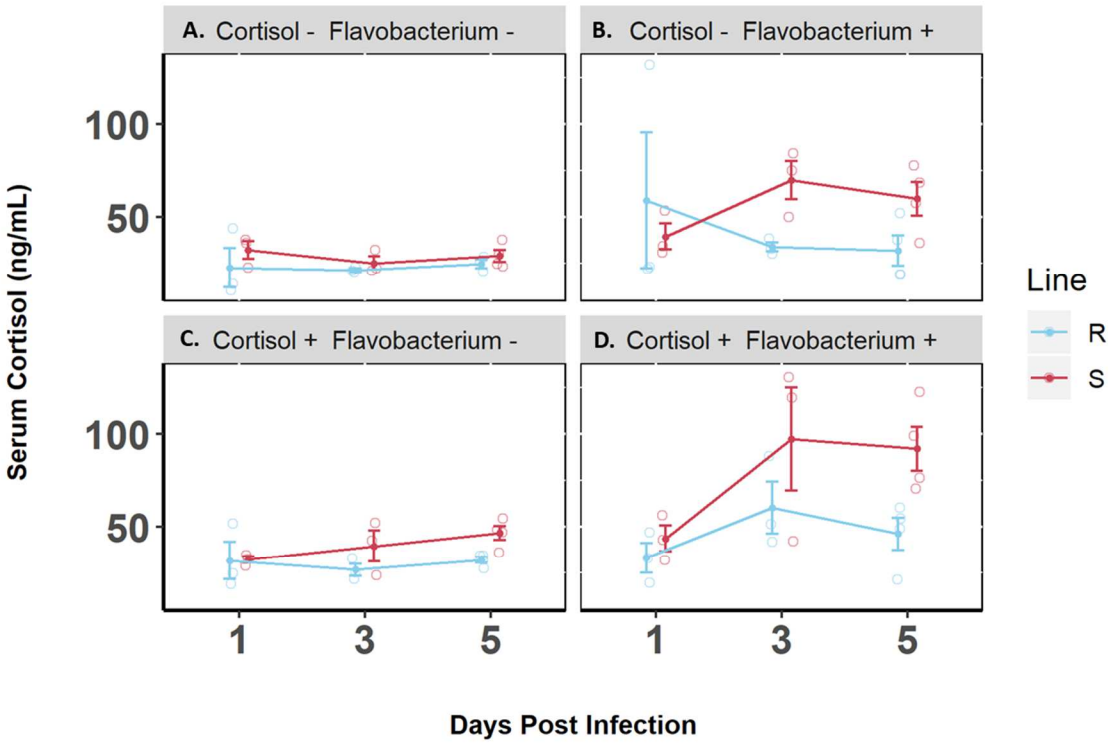

A.
S-Line
B.
R-Line
17.04
43.47
69.9
96.33
Log transformed MU Ratio
Pathogen load
Pathogen load

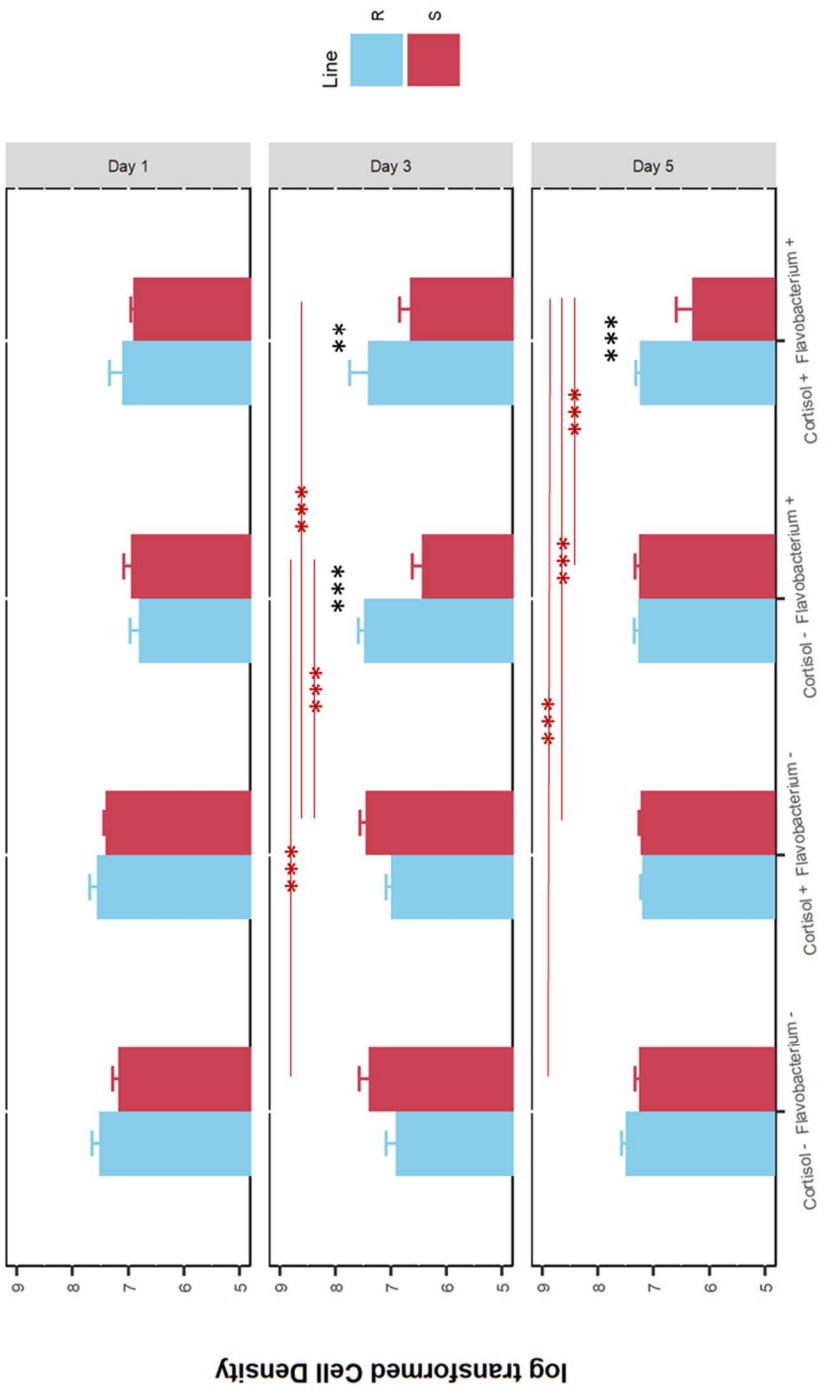
Figure 5



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

Tables

Table I: Descriptive statistics table showing mean and SD for response variables in R and S-Line in all treatment groups

A.	R-Line			Spleen Index		SPL_MU_ratio		Serum cortisol(ng/mL)		Pathogen load		Prevalence of Fp
	Group	Day	N	Mean	SD	Mean	SD	Mean	SD	Mean	SD	% fish
	C−*Fp*−	1	3	1.4	0.4	2.9	0.4	22.8	18.4	0	0	0
		3	3	1	0.1	2.6	1	21.3	1.7	0	0	0
		5	3	2.5	1.8	1.4	1.2	24.8	4.2	0	0	0
	C−*Fp*+	1	3	0.9	0.4	16.3	8.4	58.9	63.2	3.5	6.1	33
		3	3	2.2	1	2.3	0.2	33.9	4.2	4.8	8.2	33
		5	4	1.7	0.6	1.2	0.6	31.9	16.1	153.2	293.4	33
	C+*Fp*−	1	3	2.1	1.5	2	1	31.6	17.5	0	0	0
		3	3	1.5	0.9	1.7	0.5	26.5	5.8	0	0	0
		5	4	1.3	0.5	2.8	1.1	31.9	3.2	0	0	0
	C+*Fp*+	1	3	1.3	0.7	5.8	5.6	33	13.7	0	0	0
		3	3	1.5	0.2	6.9	7.3	60.3	24.3	1196.8	2053.2	100
		5	4	1.4	0.6	0.8	0.2	17.4	8.7	21.7	16.6	66
B.	**S-Line**			Spleen Index		SPL_MU ratio		Serum cortisol(ng/mL)		Pathogen load		Prevalence of Fp
	Group	Day	N	Mean	SD	Mean	SD	Mean	SD	Mean	SD	% fish
	C−*Fp*−	1	3	1.4	0.9	2.2	0.8	32.2	8.2	0	0	0
		3	3	1.4	0.3	0.6	0.4	25.2	6.1	0	0	0
		5	4	1	0.2	0.9	0.9	29	6.5	0	0	0
	C−*Fp*+	1	3	1.2	0	2.1	0.4	39.4	12.2	141.5	234.9	66
		3	3	1.5	0.3	13.6	3.7	69.8	17.7	42008	11072.1	100
		5	4	1.6	0.6	13.4	5.1	59.9	18	26.9	40.5	75
	C+*Fp*−	1	3	2.5	2.9	0.6	0.1	32.1	2.9	0	0	0
		3	3	1.6	0.9	0.4	0.1	39.4	14.4	0	0	0
		5	4	0.8	0.2	1	0.5	46.5	7.7	0	0	0
	C+*Fp*+	1	3	1	0.1	1.1	0.3	43.6	12.1	146.7	247.7	66
		3	3	1.5	0.2	3.4	2.3	97.3	48	49637.6	43016.2	100
		5	4	2	0.6	57.9	23.7	92	23.7	22141.7	27248	100

Table II: Table showing significant main and interaction effects as reported by a three-way ANOVA on Pathogen load, serum cortisol and MU ratio.

			Line (L)		Treatment Group (TG)				Days Post Infection (D)		
	Factor	P-Value	R-Line	S-Line	G1 C-Fp-	G2 C+Fp-	G3 C-Fp+	G4 C+Fp+	D1	D3	D5
A. Pathogen load											
Factors	L	<0.001	X	Y							
	TG	0.04			na	na	B	A			
	DPI	<0.001							c	a	b
Interactions	TGxL	n.s			na	na	L: S > R	L: S > R			
	TGxDPI	0.1			na	na	D: 3>1, 5>3	D: 3>1, 5>1	TG: 4>3	TG: 4>3	
	DPIxL	0.02		D: 3>1, 5>3					L: S > R	L: S > R	L: S > R
	LxDPIxTG	0.013									
B. Serum Cortisol											
Factors	L	<0.001	X	Y							
	TG	<0.001			C	BC	AB	A			
	DPI	n.s									
Interactions	LxTG	n.s									
	TGxDPI	0.1						D: 3>1		TG: 4>1&2	
	LxDPI	0.07		D: 5>1, 3>1						L: S>R	L: S>R
	LxDPIxTG	n.s									
C. Spleen Mu Ratio											
Factors	L										
	TG	<0.001			B	B	A	A			
	DPI										
Interactions	LxTG	<0.001		TG: 3&4>1, 2>3, 4>2	L: S>R	L: S>R	L: S>R	L: S>R			
	TGxDPI	<0.001			D: 5>1			D: 5>1, 5>3	TG: 2>3	TG: 2>1&3, 4>1&2	TG:3>1, 4>1&2
	LxDPI	<0.001	D: 5>1, 5>3	D: 5>1, 3>1					L: S>R	L: S>R	L: S>R
	LxDPIxTG	<0.001									

Table III. mRNA transcript fold change values and ANOVA summary table for membrane and secreted forms of mu and tau as measured in AK

A.	memHCmu			memHCtau			secHCmu			secHCtau		
Effect	df	F value	P value	df	F value	P value	df	F value	P value	df	F value	P value
L	1	5.04	**0.03**	1	0.36	0.55	1	9.8	**0**	1	0.43	0.51
TG	3	6.74	**0**	3	4.41	**0.01**	3	1.93	0.14	3	0.81	0.5
DPI	2	1.38	0.26	2	0.26	0.77	2	2.72	0.08	2	0.4	0.67
TGxL	3	3.11	**0.03**	3	1.3	0.29	3	1.29	0.29	3	1.89	0.14
TGxDPI	6	3.57	**0**	6	1.5	0.2	6	2.53	**0.03**	6	1.29	0.28
DPIxL	2	1.9	0.16	2	2.21	0.12	2	2.74	**0.07**	2	0.29	0.75
LxDPIxTG	6	0.9	0.5	6	0.8	0.58	6	1.57	0.17	6	0.96	0.46

B.		Cortisol - Flavobacterium -				Cortisol - Flavobacterium +				Cortisol + Flavobacterium -				Cortisol + Flavobacterium +			
		R-Line		S-Line		R-Line		S-Line		R-Line		S-Line		R-Line		S-Line	
	Days	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
memHCmu	1	2.1	0.7	1.1	0.3	2.6	1.2	3.5	2	0.6	0.2	1.5	0.4	1.3	0.2	0.8	0.4
	3	1.2	0.2	3.6	1.5	0.7	0.3	0.6	0	0.6	0.1	6.5	3	0.6	0.2	0.7	0.1
	5	2.9	0.1	4	1	0.8	0.4	0.9	0.2	1.8	0.6	2.5	0.8	0.9	0.1	1.2	0.4
secHCmu	1	1.2	0.3	1.1	0.3	2	0.7	3	0.8	0.9	0.4	1.9	0.5	2.1	0.3	0.9	0.4
	3	1.2	0.7	3	0.9	0.8	0.3	1	0.1	0.7	0.3	7.1	2	0.9	0.3	1.4	0.4
	5	2.9	0.6	6.1	1.5	1.2	0.5	2.2	0.9	3.1	1.1	2.8	1	1	0.1	2.7	1
memHCtau	1	1.1	0.3	1.2	0.5	2.1	1	0.6	0.3	0.2	0	0.6	0.3	0.5	0.2	0.4	0.3
	3	1.3	0.4	1.7	0.8	0.3	0.1	0.3	0.1	0.2	0.1	2	0.6	0.1	0.1	0.4	0.3
	5	1.3	0.8	1	0.6	0.3	0.1	0.4	0.2	1.3	0.7	1.7	0.7	0.4	0.2	0.8	0.6
secHCtau	1	4.4	2.1	1.3	0.7	1.9	0.8	1.6	1.2	1	0.3	0.8	0.6	0.8	0.2	3.9	2.6
	3	10.2	7.8	1.1	0.5	0.5	0.2	1.5	0.8	1.2	0.8	1.2	0.3	0.3	0.2	0.7	0.4
	5	7.4	7.2	0.9	0.5	0.8	0.4	2.8	1.7	15.8	9.9	1.5	0.8	0.6	0.2	5.5	4.5

Table IV. Summary tables for linear models showing the combined effect of cortisol and pathogen load on MU ratio for both lines.

	Call: lm(SPL_MU_ratio ~ Average_cortisol * Pathogen_load)					
A.	R-Line					
	Coefficients:					
		Estimate	SE	t value	Pr(>|t|)	
	(Intercept)	0.41	0.07	5.89	0.00	***
	Average_cortisol	-0.01	0.01	-1.06	0.30	
	Pathogen_load	0.33	0.17	1.90	0.07	.
	Average_cortisol: Pathogen_load	-0.03	0.01	-2.56	0.02	*
	Residual standard error: 0.335 on 32 degrees of freedom					
	Multiple R-squared: 0.2076, Adjusted R-squared: 0.1333					
	F-statistic: 2.794 on 3 and 32 DF, p-value: 0.0561					
B.	S-Line					
		Estimate	SE	t value	Pr(>|t|)	
	(Intercept)	0.50	0.10	5.00	0.00	***
	Average_cortisol	0.02	0.00	3.05	0.00	**
	Pathogen_load	0.19	0.06	2.96	0.01	**
	Average_cortisol: Pathogen_load	0.00	0.00	-2.25	0.03	*
	Residual standard error: 0.4796 on 35 degrees of freedom					
	Multiple R-squared: 0.6099, Adjusted R-squared: 0.5533					
	F-statistic: 2.794 on 3 and 32 DF, p value: 0.0561					

Chapter 3

Concluding Remarks

We have presented in this dissertation a new approach to investigate the basis of *Flavobacterium psycrophilum* resistance in rainbow trout. To this end, we investigated the immunoglobulin gene expression changes (Ig response) to a slow release cortisol implant followed by *Fp*-challenge in a *Fp*-Resistant and control (*Fp*-Susceptible) line of Rainbow trout. As has been shown by others, and in agreement with our data, there is differential disease progression among the two lines (Marancik et al., 2014). Our experimental approach allows us to work around the base mechanisms used by these lines to eliminate the pathogen. Inducing stress to increase pathogen load in the R-line makes the immune response comparable to S-line; this allows us to study the immune response to identify the differential pathogen clearance mechanisms between R and S-line.

We report that in S-line fish, there is high mobilization of IgM++ ISCs in the blood on day 1 post infection which is not observed on day 3 and 5. Concurrently, on day 3 and 5, a higher MU ratio is observed in the spleen. We can speculate that by day 1, existing IgM+ plasma cells are mobilized in response to *Fp* infection; they circulate in the blood to reach potential target sites such as the muscles. An earlier study from our lab shows that S-line fish have a higher 'basal' level of IgM circulating in the blood (Zwollo et al., 2015). However, we did not see an increase in the Membrane or secreted mu mRNA transcripts in the anterior kidney. We can further speculate that new B-cells are not formed in response to the infection until late stages. In comparison, we don't see a mobilization of ISCs in R-line post challenge in blood. Consequently, the IgM upregulation in spleen is also lower. Furthermore, we also observe a differential IgM response post *Fp*-challenge only in R and S-line on day 1, when the pathogen load is

similar in both lines. This might indicate an efficacious protective response of IgM in R-line as compared to S-line due to stronger affinity of antibodies. It also might also indicate a difference in clearance mechanisms by both lines; non-specific immunity might play an important role in R-line fish.

Several studies have looked at how the non-specific immune genes are modulated after either an experimental *Fp*-challenge or slow release cortisol implants. As investigated by two high throughput transcriptomic studies after an *Fp*-challenge, differentially regulated innate immune genes generally can be categorized in a few major categories; cytokines and chemokines (IL-1B, IL-10 and CC chemokine upregulated in S-line; (Langevin et al., 2012; Marancik et al., 2015), acute phase proteins (SAA and lysozyme II, induced in R-line but levels did not reach basal S-line levels; (Langevin et al., 2012) and complement system components (C3; (Langevin et al., 2012) and complement factor H upregulated; (Marancik et al., 2015) in R-line). Other immune relevant genes include Eosinophil-peroxidase-like (expressed on neutrophil like cells involved in phagocytosis) (Marancik et al., 2015) and M-CSFR (Moore et al., 2019) upregulated in R-line after infection and SOCS which has higher basal expression in the S-line (Langevin et al., 2012). (Moore et al., 2019) also suggests protective role of Macrophages/monocytic populations as measured through flow cytometry in resistance of R-line (Moore et al., 2019). Justifiably, higher IL-10, involved in inhibition of phagocytosis through macrophages, and higher SOCS, involved in suppression of cytokines, might predispose S-line to a worse disease progression. In contrast, in R-line; Eosinophil-peroxidase-like, complement factors and M-CSFR points towards enhanced phagocytic ability that could lead to clearance of pathogen by non-specific immunity and hence better disease progression. In the studies reported above, gene expression differences between lines are observed on later days after infection when the disease

has already progressed differentially. This is in agreement to our findings, where we also report significant change in MU ratio between lines on day 5.

In our experimental design, we injected slow release cortisol implants, in addition to the *Fp*-challenge, to examine how the resistance was affected and whether high endogenous cortisol levels could break that resistance by inducing high pathogen loads. Several studies have reported on the effects of cortisol implants on immune genes; however, to our knowledge, no studies have looked at differential regulation of immune genes between lines after cortisol implant. (Cortés et al., 2013) detected low complement and lysozyme activity and downregulation of complement factor c3 and IL-1b in the anterior kidney. IL-1b was reported to be upregulated in spleen (Cortés et al., 2013) and liver (Philip et al., 2012) after cortisol implant. Another interesting study, similar to the current study, which studied innate immune genes after cortisol implant followed by IHNV challenge in RBT reported higher pathogen loads in fish with cortisol implants.

It is very interesting to see that similar genes are being differentially modulated after cortisol implant and *Fp*-challenge separately. In our study, we report differential regulation of endogenous cortisol levels in response to cortisol implants followed by *Fp*-challenge. However, by observing individual data points for increase in endogenous cortisol levels, we infer serum cortisol levels might play an important biological role in downstream processes such as pathogen clearance and eliciting immune response. The statistical non-significance might be due to very low sample size (n=3 on each day). As aforementioned, the pathogen load and serum cortisol levels are highest in cortisol-implanted and *Fp*-challenged treatment group in both genetic lines; S-line being higher. Although we cannot determine cause and effect, it is clear that in S-line, the IgM response is exacerbated in cortisol implanted fish; this might indicate the fish entering a

vicious cycle of increased immune response due to consistently multiplying pathogen. As indicated in SARS-CoV-2 research, higher inflammation with continuous replication of virus leads to acute damage in the lungs which is the cause of mortality in many patients. We think S-line fish might undergo a similar exacerbation and inflammatory response leading to mortality (Fu et al., 2020).

Another interesting experimental design could include either blocking the cortisol receptors or using an antagonist against cortisol in RBT and then challenging them with *Fp*. This might impart resistance to S-line and scientists would be able to determine cause and effect. Interestingly, in spleen and liver, after cortisol implants, the GR receptors are downregulated (Gadan et al., 2012; Teles et al., 2013). It would be interesting to quantify glucocorticoid receptors in R and S-line and see how their expression levels change after *Fp*-challenge with cortisol implant.

In this century, stress has come to be known as a major factor that drives behaviors and actions. Mental health is taken more seriously, and more studies are now looking at the effect of stress on a molecular level. Although current study targets a very specific area of immunology, it would have significant broader impacts. For example, Sephton et al. (Sephton et al., 2009) shows that cortisol levels, depression and suppressed cell-mediated immunity are co-related in metastatic breast cancer. Current study can help us to better understand the effect of stress on immune system; it can be useful in auto-immune diseases and mental health diseases as well.

Finally, it may be concluded that cortisol is an important regulator of immune response and might play a substantial role in disease progression in genetic lines of RBT. Furthermore, a stronger affinity antibody response might be a key player in enhancing phagocytosis and removal of *Fp* to stop the disease from spreading to systemic organs in R-line fish.

In a field study on sockeye salmon returning to their natal grounds to spawn (Smith and Zwollo), it has been shown that fish caught in the middle of the spawning journey, have an increased abundance of IgM++ cells compared to fish caught at the mouth of the river or the spawning grounds (Smith; unpublished data). Although cortisol levels were not measured in this study, another study that investigated migration from river to sea after successful spawning in Atlantic salmon reports that the fish with higher plasma cortisol levels exit the river early and are less likely to survive the migration journey (Birnie-Gauvin et al., 2019). On a similar note, another study reported that fish with higher cortisol levels might not survive the hydraulic challenges faced during the migration journey (Twardek et al., 2019). In summary, it can be said, with caution, that migrating fish that activate their immune response despite high levels of cortisol might suffer pre-spawning mortality. On the one hand, one could argue that newly generated ISCs provide antibody protection against pathogens that were not encountered as juveniles, (eg newly emerged pathogens at their natal ground). On the other hand, strong antibody responses late in the journey may be detrimental at this stage of a salmon's' life cycle (such as S-line in the current study). Most if not all fish have high pathogen loads by the time they reach their spawning grounds, as reported by us and others. Perhaps those fish that respond to pathogens with strong antibody responses are more likely to suffer pre-spawning mortality from excessive (antibody-dependent enhancement) of inflammation, at a time where it needs to use most of its energy towards completing the spawning process, before dying (i.e. S-line fish in the current study). Perhaps, the migrating fish that are genetically more similar to R-line are the ones that successfully complete their migration journey. Hence, the question of optimal antibody protection during the spawning journey remains elusive. Therefore perhaps, out of the two hypothesis we stated earlier, the second one ‘Salmon that activated their immune system after encountering pathogens were unable to fight the infection which

led to early mortality. The only subset of migrating salmon that reached the spawning grounds were those that were able to combat the pathogens without activating the antibody response' makes more sense, although due to several other factors involved, this hypothesis should be taken forward with caution.

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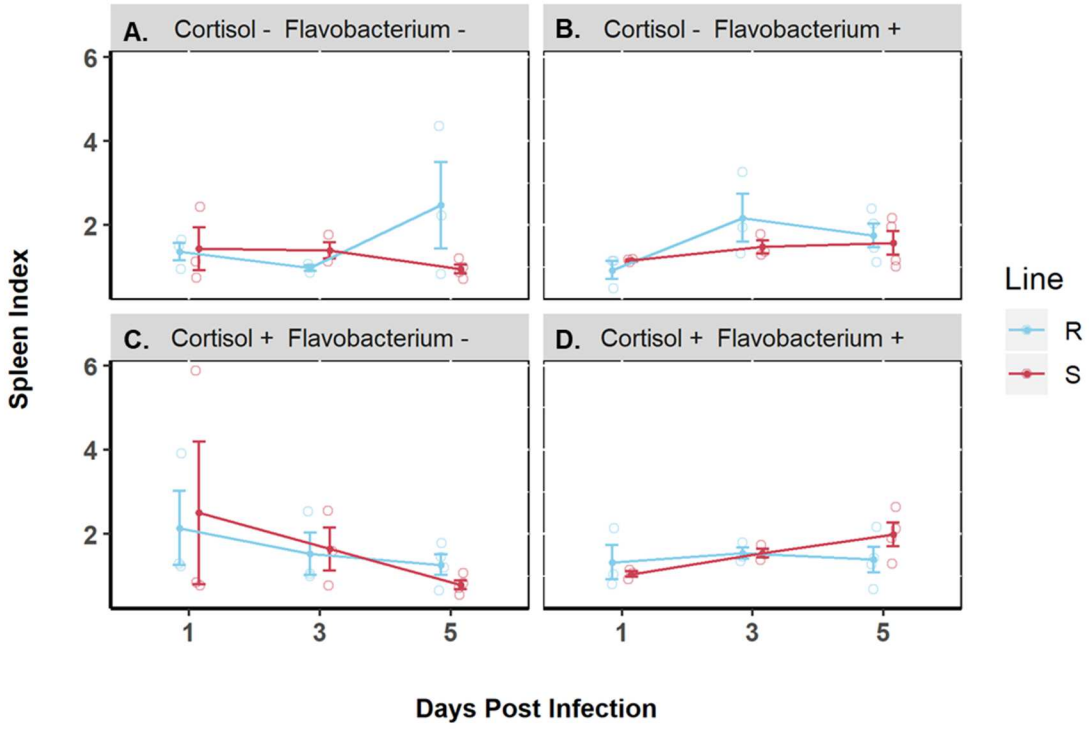
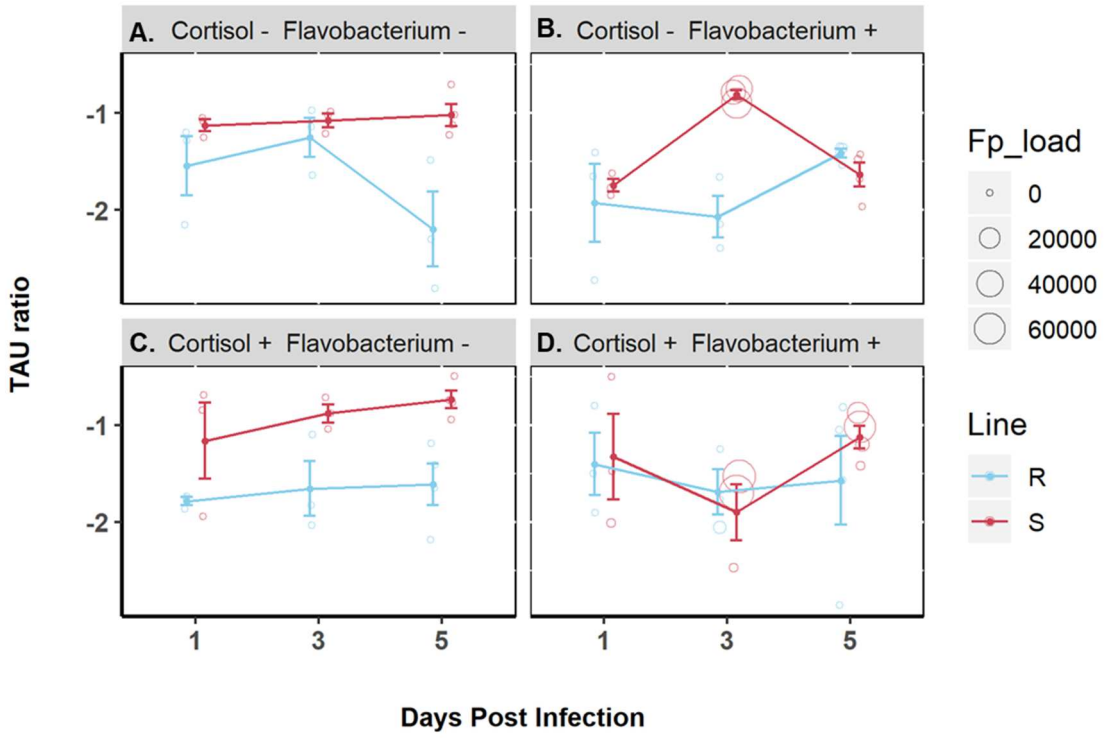
Appendices

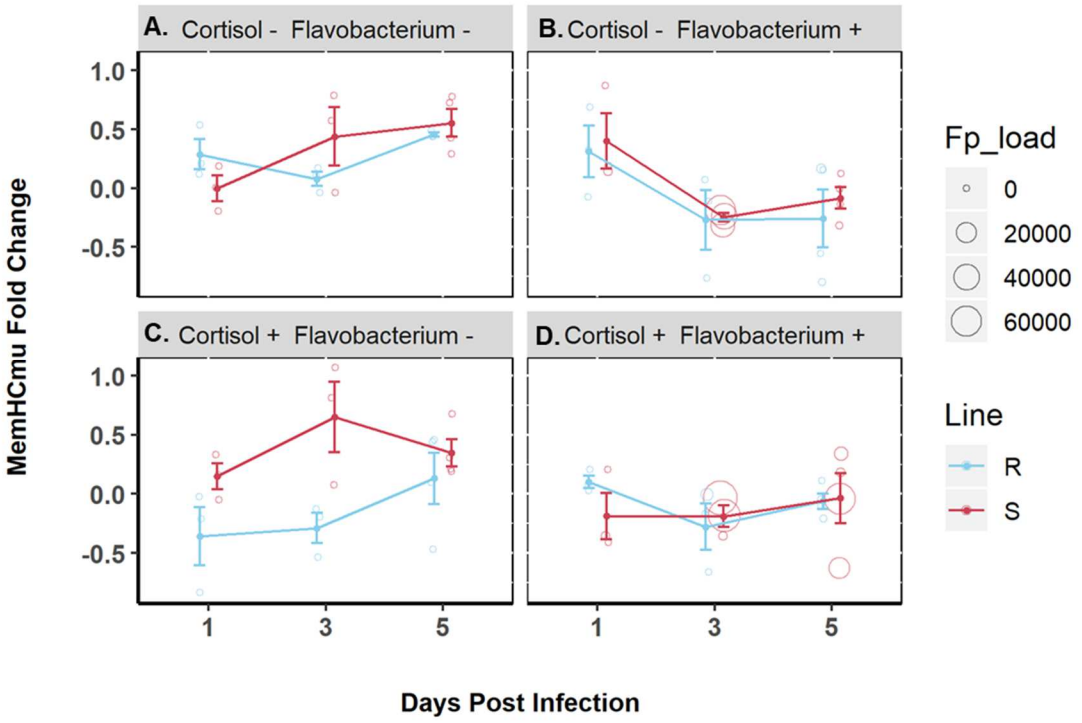
A1. Figures explained but not included in the Manuscript



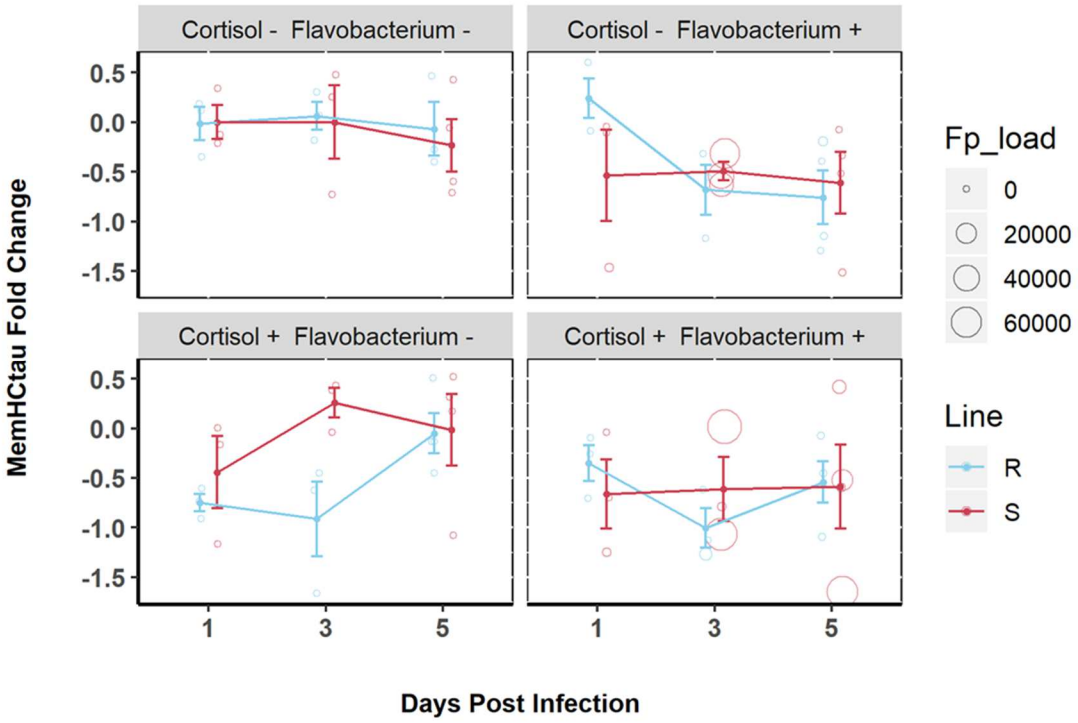
Appendix Figure 1. Strip charts showing Spleen Index (spleen wt. relative to body wt. in grams) (A.) and cortisol implanted *Fp* challenged fish (B.). The Y-axis shows Spleen index values. The X-axis shows Days Post Infection. Individual data points (open circles), average values, and standard error bars for R-line are shown in blue and for S-line is shown in red. No significance was observed between groups. **A.** Mock cortisol implants with mock *Fp* challenge; **B.** Mock cortisol implants with *Fp* challenge; **C.** Cortisol implants with *Fp* mock challenge; **D.** Cortisol implants with *Fp* challenge.



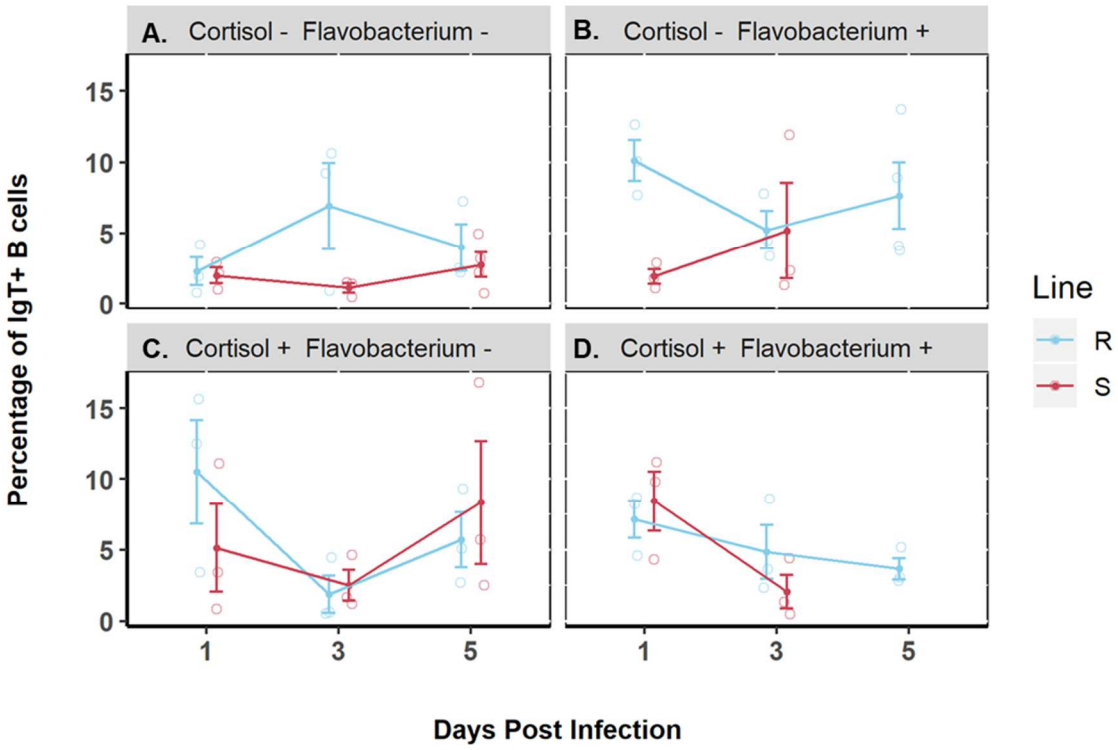
Appendix Figure 2. Strip charts showing IgT response denoted by the ratio of secreted to membrane form of Heavy chain tau (TAU ratio) on different days post challenge. The Y-axis shows log transformed fold change value of TAU ratio in spleen. The X-axis shows Days Post Infection. Individual data points (open circles), average values and standard error bars for R-line are shown in blue and for S-line is shown in red. Size of open circles represents copy number of *Fp* as described in the figure legend. No significance was observed between groups for TAU ratio. **A.** Mock cortisol implants with mock *Fp* challenge; **B.** Mock cortisol implants with *Fp* challenge; **C.** Cortisol implants with *Fp* mock challenge; **D.** Cortisol implants with *Fp* challenge.



Appendix Figure 3. Strip charts showing MemHCmu fold change values as measured through mRNA expression in anterior kidney on different days post challenge. The Y-axis shows log transformed fold change value of memHCmu in anterior kidney. The X-axis shows Days Post Infection. Individual data points (open circles), average values and standard error bars for R-line are shown in blue and for S-line is shown in red. Size of open circles represents copy number of *Fp* as described in the figure legend. No significance was observed between groups. **A.** Mock cortisol implants with mock *Fp* challenge; **B.** Mock cortisol implants with *Fp* challenge; **C.** Cortisol implants with *Fp* mock challenge; **D.** Cortisol implants with *Fp* challenge.



Appendix Figure 4. Strip charts showing MemHCtau fold change values as measured through mRNA expression in anterior kidney on different days post challenge. The Y-axis shows log transformed fold change value of memHCtau in anterior kidney. The X-axis shows Days Post Infection. Individual data points (open circles), average values and standard error bars for R-line are shown in blue and for S-line is shown in red. Size of open circles represents copy number of *Fp* as described in the figure legend. No significance was observed between groups. **A.** Mock cortisol implants with mock *Fp* challenge; **B.** Mock cortisol implants with *Fp* challenge; **C.** Cortisol implants with *Fp* mock challenge; **D.** Cortisol implants with *Fp* challenge.



Appendix Figure 5. Strip charts showing the abundance of IgT+/IgM- B cells circulating in Blood from flow cytometry data. The Y-axis shows abundance of IgT+/IgM- B cells (in percentage cells). The X-axis shows Days Post Infection. Individual data points (open circles), average values (solid circles), and standard error bars for R-line are shown in blue and for S-line is shown in red color. No significance was observed between groups. Day 5 had missing data points for S-line and thus, has not been shown in the figure. **A.** Mock cortisol implants with mock challenge; **B.** Mock cortisol implants with *Fp* challenge; **C.** Cortisol implants with mock challenge; **D.** Cortisol implants with *Fp* challenge.

A2. Example code for generating Strip Charts

Spl_MU_ratio_strip_chart.R

Fatima Quddos

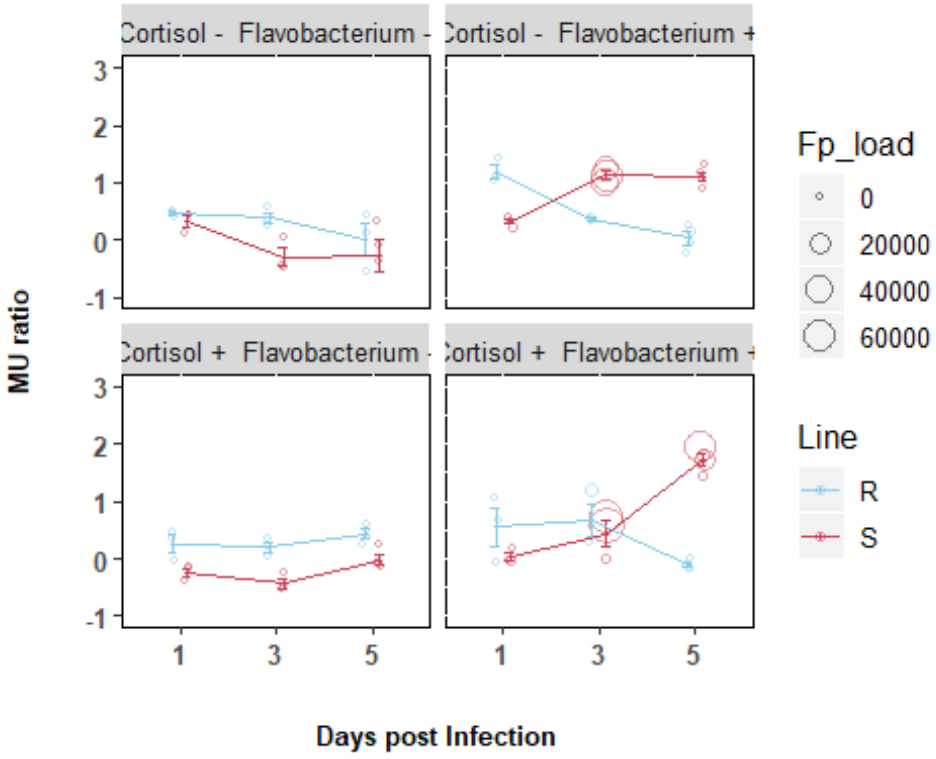
2020-06-17

```
#Fatima Q.
#27 July, 2019 #Fp 2019
#This code is for making strip charts for SPL_MU_ratio

#Libraries for everything you need
library(dplyr)

##
## Attaching package: 'dplyr'

## The following objects are masked from 'package:stats':
##
##     filter, lag

## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union

library(ggplot2)
library(Hmisc)

## Loading required package: lattice

## Loading required package: survival

## Loading required package: Formula

##
## Attaching package: 'Hmisc'

## The following objects are masked from 'package:dplyr':
##
##     src, summarize

## The following objects are masked from 'package:base':
##
##     format.pval, units

library(gridExtra)
```

```
##
## Attaching package: 'gridExtra'

## The following object is masked from 'package:dplyr':
##
##     combine

library(tidyr)

## Warning: package 'tidyr' was built under R version 3.6.2

library(readxl)

#clear R's brain
rm(list = ls())
#Import dataset
Fp_Data <- read_excel("Fp2019_Cortisol_v2.xlsx")

#Function to calculate mean and sd
summarySE <- function(data=NULL, measurevar, groupvars=NULL, na.rm=FALS
E,
                      conf.interval=.95, .drop=TRUE) {
  library(plyr)

  # New version of length which can handle NA's: if na.rm==T, don't cou
nt them
  length2 <- function (x, na.rm=FALSE) {
    if (na.rm) sum(!is.na(x))
    else       length(x)
  }

  # This does the summary. For each group's data frame, return a vector
with
  # N, mean, and sd
  datac <- ddply(data, groupvars, .drop=.drop,
                 .fun = function(xx, col) {
                   c(N    = length2(xx[[col]], na.rm=na.rm),
                     mean = mean   (xx[[col]], na.rm=na.rm),
                     sd   = sd     (xx[[col]], na.rm=na.rm)
                   )
                 },
                 measurevar
  )

  # Rename the "mean" column
  datac <- rename(datac, c("mean" = measurevar))

  datac$se <- datac$sd / sqrt(datac$N)  # Calculate standard error of t
he mean

  # Confidence interval multiplier for standard error
```

```
  # Calculate t-statistic for confidence interval:
  # e.g., if conf.interval i s .95, use .975 (above/below), and use df=
N-1
  ciMult <- qt(conf.interval/2 + .5, datac$N-1)
  datac$ci <- datac$se * ciMult

  return(datac)
}

posn.d <- position_dodge(width=0.3)
posn.jd <- position_jitterdodge(jitter.width = 0.05, dodge.width = 0.3)
posn.j <- position_jitter(width = 0.3)

# Making a new column with Treatment_groups
Fp_Data_united<-separate(Fp_Data, col= "Group_name", into= c("New_group
s", "2nd group", "3rd group"), sep= "_")
Fp_Data_united2<-unite(Fp_Data_united, '2nd group', '3rd group', col =
"Group_names", sep= "_")
Fp_Data <- cbind(Fp_Data, Fp_Data_united2["Group_names"])
# Data Transformation
Fp_Data$SPL_Sec_mem_mu_Ratio <- log10(Fp_Data$SPL_Sec_mem_mu_Ratio)
Fp_Data$Fp_load = Fp_Data$BCWD_mean

Fp_Data <- Fp_Data %>%
  mutate(
    Group_names = case_when (Group_names == "C0_Fp0" ~ "Cortisol -  Fla
vobacterium -",
                             Group_names == "C1_Fp0" ~ "Cortisol +  Fla
vobacterium -",
                             Group_names == "C1_Fp1" ~ "Cortisol +  Fla
vobacterium +",
                             Group_names == "C0_Fp1" ~ "Cortisol -  Fla
vobacterium +"))

Group1stats <- summarySE(Fp_Data, measurevar="SPL_Sec_mem_mu_Ratio",gro
upvars= c("Group_name", "Day", "Line","Group_names"))

as.factor(Group1stats$Day)

##  [1] 1 3 5 1 3 5 1 3 5 1 3 5 1 3 5 1 3 5 1 3 5 1 3 5
## Levels: 1 3 5

First <- ggplot()+
  geom_point(data= Fp_Data,aes(x=as.factor(Fp_Data$Day),y=SPL_Sec_mem_m
u_Ratio, col = Line, size = Fp_load), shape = 1,  position = posn.jd, a
lpha = 0.5)+
  geom_errorbar(data=Group1stats,aes(x = as.factor(Group1stats$Day), ym
in=SPL_Sec_mem_mu_Ratio-se,ymax=SPL_Sec_mem_mu_Ratio+se, col = Line), w
idth = 0.2,  position = posn.d)

Third <- First+
```

```
  geom_point(data = Group1stats,  aes(x = as.factor(Group1stats$Day), y
= SPL_Sec_mem_mu_Ratio, color = Line), shape = 20, position = posn.d)+
  geom_line (data = Group1stats ,aes(x = as.factor(Group1stats$Day), y=
SPL_Sec_mem_mu_Ratio, color = Line, group = Group_name ), position = po
sn.d)

FinalGraph=Third +xlab("Days post Infection") + ylab("MU ratio") + scal
e_color_manual(values = c("skyblue", "#cb4154"))
Adjust=FinalGraph+theme(panel.background = element_rect(fill = 'white',
colour = 'black'))

science_theme = theme(
  axis.line = element_line(size = 0.7, color = "black"),
  text = element_text(size = 12),
  plot.title = element_text(hjust=0.5))

Plot2 <- Adjust +
  science_theme

Final= Plot2+theme(axis.title.x=element_text(face="bold",size=10))+them
e(axis.title.y=element_text(face="bold",size=10))+theme(axis.text.x=ele
ment_text(size=10,face="bold"))+theme(axis.text.y=element_text(size=10,
face="bold"))+theme(plot.title=element_text(face="bold",size=20))
Plot3 <- Final+theme(axis.title.y=element_text(margin=margin(0,20,0,0))
)+theme(axis.title.x=element_text(margin=margin(20,0,0,0)))

Final_plot <- Plot3 + facet_wrap( ~Group_names) +ylim(-1, 3)

Final_plot
```




```
ggsave("Final_SPL_MU_Ratio_strip_chart.png", device = "png", width= 6,
height = 4,
       Final_plot)
```

A3. Linear modelling code

Linear-model.R

Fatima Quddos

2020-06-17

```
#Fatima Q.
#Fp 2019
#This code is for the analysis of multiple regression between MU_Ratio
and Pathogen load*Cortisol levels

library(dplyr)

##
## Attaching package: 'dplyr'
```

```
## The following objects are masked from 'package:stats':
##
##     filter, lag

## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union

library(tidyr)

## Warning: package 'tidyr' was built under R version 3.6.2

library(ggplot2)
library(Hmisc)

## Loading required package: lattice

## Loading required package: survival

## Loading required package: Formula

##
## Attaching package: 'Hmisc'

## The following objects are masked from 'package:dplyr':
##
##     src, summarize

## The following objects are masked from 'package:base':
##
##     format.pval, units

library(gridExtra)

##
## Attaching package: 'gridExtra'

## The following object is masked from 'package:dplyr':
##
##     combine

library(ggpubr)

## Loading required package: magrittr

##
## Attaching package: 'magrittr'

## The following object is masked from 'package:tidyr':
##
##     extract

library(readxl)
library(car)
```

```
## Warning: package 'car' was built under R version 3.6.2

## Loading required package: carData

##
## Attaching package: 'car'

## The following object is masked from 'package:dplyr':
##
##     recode

library(nlme)

##
## Attaching package: 'nlme'

## The following object is masked from 'package:dplyr':
##
##     collapse

library(effects)

## Warning: package 'effects' was built under R version 3.6.3

## Registered S3 methods overwritten by 'lme4':
##   method                          from
##   cooks.distance.influence.merMod car
##   influence.merMod                car
##   dfbeta.influence.merMod         car
##   dfbetas.influence.merMod        car

## Use the command
##     lattice::trellis.par.set(effectsTheme())
##   to customize lattice options for effects plots.
## See ?effectsTheme for details.

library(psych)

## Warning: package 'psych' was built under R version 3.6.3

##
## Attaching package: 'psych'

## The following object is masked from 'package:car':
##
##     logit

## The following object is masked from 'package:Hmisc':
##
##     describe
```

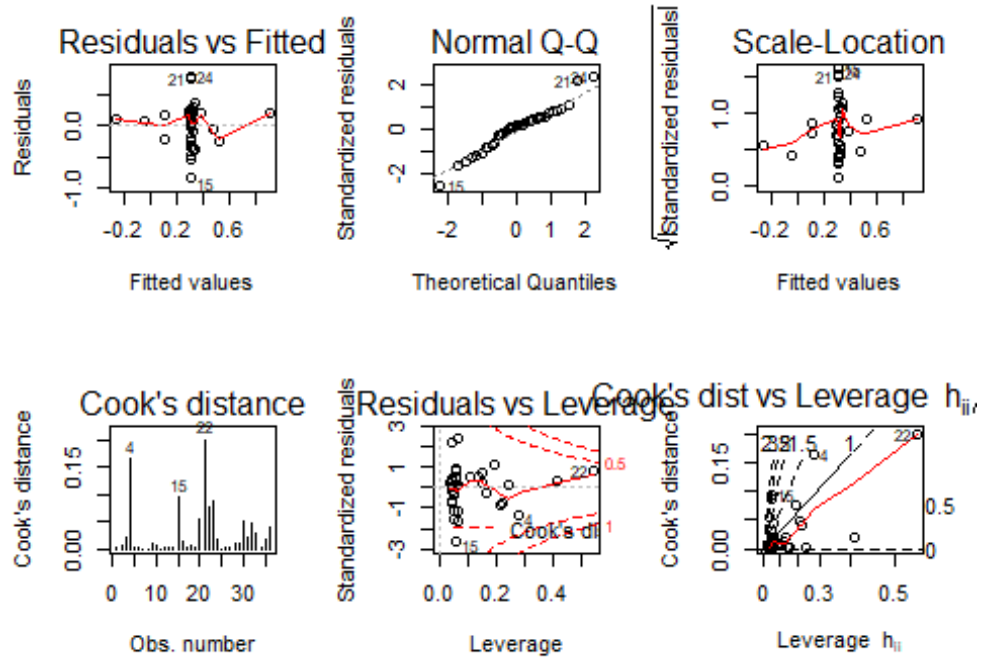
```
## The following objects are masked from 'package:ggplot2':
##
##     %+%, alpha

library(multcompView)

## Warning: package 'multcompView' was built under R version 3.6.3

library(GGally)

## Warning: package 'GGally' was built under R version 3.6.3

## Registered S3 method overwritten by 'GGally':
##   method from
##   +.gg   ggplot2

##
## Attaching package: 'GGally'

## The following object is masked from 'package:dplyr':
##
##     nasa

rm(list = ls())
Fp_Data <- read_excel("Fp2019_Cortisol_v2.xlsx") #Import dataset

#Cleaning Data
Fp_Data_united<-separate(Fp_Data, col= "Group_name", into= c("New_group
s", "2nd group", "3rd group"), sep= "_")
Fp_Data_united2<-unite(Fp_Data_united, '2nd group', '3rd group', col =
"Treatment_Groups", sep= "_")
Fp_Data <- cbind(Fp_Data, Fp_Data_united2["Treatment_Groups"])
Fp_Data$Line = as.factor(Fp_Data$Line)
Fp_Data$Day = as.factor (Fp_Data$Day)
Fp_Data$Treatment_Groups = as.factor(Fp_Data$Treatment_Groups)

#Data Transformation
Fp_Data$SPL_MU_ratio = log10(Fp_Data$SPL_Sec_mem_mu_Ratio)## Transforma
tion of data
Fp_Data$SPL_TAU_ratio = log10(Fp_Data$SPL_Sec_mem_tau_Ratio)
Fp_Data$Pathogen_load = log10(Fp_Data$BCWD_mean+1)
Fp_Data$Serum_cortisol_levels = Fp_Data$Average_cortisol

Fp_Data_Fpp <- Fp_Data %>%
  filter (Fp == 1)


Fp_Data_R <- Fp_Data %>%
  filter(Line == "R")

Fp_Data_S <- Fp_Data %>%
  filter(Line == "S")
```

```r
Fp_Data_R_rem <- Fp_Data_R[-c(31,20,34),]

Fp_Data_cor <- Fp_Data %>%
  select(Spleen_index, Serum_cortisol_levels, Pathogen_load, SPL_MU_rat
io, SPL_TAU_ratio, Line)

Fp_Data_cor_R <- Fp_Data_cor %>%
  filter(Line == "R")

Fp_Data_cor_S <- Fp_Data_cor %>%
  filter(Line == "S")

#Modeling

r.lm.rem <- lm ( SPL_MU_ratio ~ scale(Average_cortisol, scale = FALSE)*
scale(Pathogen_load, scale = FALSE), data = Fp_Data_R_rem)
summary(r.lm.rem)

##
## Call:
## lm(formula = SPL_MU_ratio ~ scale(Average_cortisol, scale = FALSE) *
##     scale(Pathogen_load, scale = FALSE), data = Fp_Data_R_rem)
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -0.84493 -0.20951  0.05167  0.17314  0.77183
##
## Coefficients:
##
Estimate
## (Intercept)
0.407584
## scale(Average_cortisol, scale = FALSE)
-0.006201
## scale(Pathogen_load, scale = FALSE)
0.329938
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE) -0.025754
##
Std. Error
## (Intercept)
0.069222
## scale(Average_cortisol, scale = FALSE)
0.005862
## scale(Pathogen_load, scale = FALSE)
0.173284
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE)   0.010045
##
```

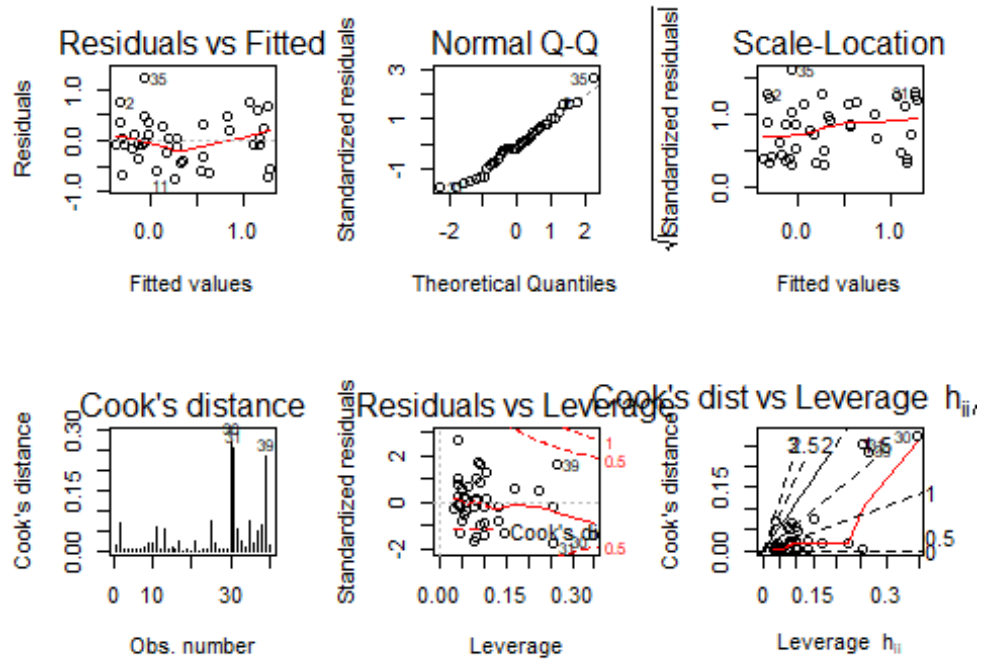
```
t value
## (Intercept)
5.888
## scale(Average_cortisol, scale = FALSE)
-1.058
## scale(Pathogen_load, scale = FALSE)
1.904
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE)  -2.564
##
Pr(>|t|)
## (Intercept)
1.5e-06
## scale(Average_cortisol, scale = FALSE)
0.2980
## scale(Pathogen_load, scale = FALSE)
0.0659
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE)   0.0152
##
## (Intercept)
***
## scale(Average_cortisol, scale = FALSE)
## scale(Pathogen_load, scale = FALSE)
.
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE) *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.335 on 32 degrees of freedom
## Multiple R-squared:  0.2076, Adjusted R-squared:  0.1333
## F-statistic: 2.794 on 3 and 32 DF,  p-value: 0.0561

s.lm <- lm( SPL_MU_ratio ~ scale(Average_cortisol, scale = FALSE)*scale
(Pathogen_load, scale = FALSE),
            data = Fp_Data_S)
summary(s.lm)

##
## Call:
## lm(formula = SPL_MU_ratio ~ scale(Average_cortisol, scale = FALSE) *
##     scale(Pathogen_load, scale = FALSE), data = Fp_Data_S)
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -0.76102 -0.34145 -0.05559  0.28866  1.21816
##
## Coefficients:
##
```

```
Estimate
## (Intercept)
0.497660
## scale(Average_cortisol, scale = FALSE)
0.015230
## scale(Pathogen_load, scale = FALSE)
0.187215
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE) -0.003853
##
Std. Error
## (Intercept)
0.099597
## scale(Average_cortisol, scale = FALSE)
0.004997
## scale(Pathogen_load, scale = FALSE)
0.063266
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE)   0.001715
##
t value
## (Intercept)
4.997
## scale(Average_cortisol, scale = FALSE)
3.047
## scale(Pathogen_load, scale = FALSE)
2.959
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE)  -2.246
##
Pr(>|t|)
## (Intercept)
1.52e-05
## scale(Average_cortisol, scale = FALSE)
0.00431
## scale(Pathogen_load, scale = FALSE)
0.00543
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE)  0.03093
##
## (Intercept)
***
## scale(Average_cortisol, scale = FALSE)
**
## scale(Pathogen_load, scale = FALSE)
**
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE) *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

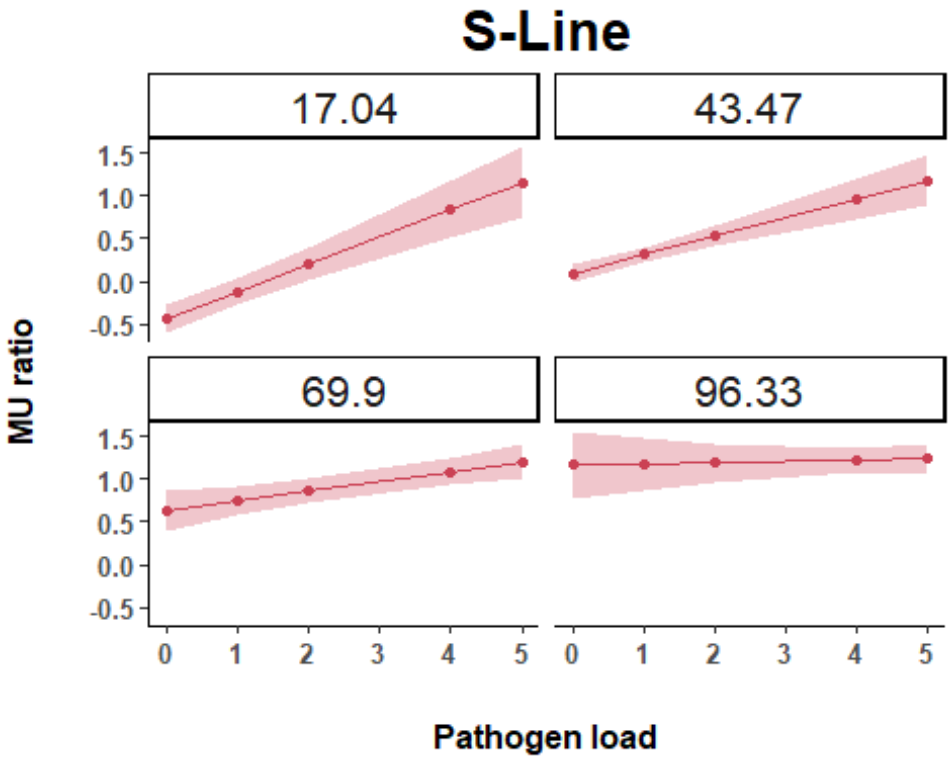
```
##
## Residual standard error: 0.4729 on 36 degrees of freedom
## Multiple R-squared:  0.6009, Adjusted R-squared:  0.5677
## F-statistic: 18.07 on 3 and 36 DF,  p-value: 2.539e-07

### Making sure assumptions are met
vif(s.lm) # Testing for multicolinearity

##                                     scale(Average_cortisol, scale =
FALSE)
##                                                                3.
469544
##                                        scale(Pathogen_load, scale =
FALSE)
##                                                                2.
367056
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE)
##                                                                2.
420000

vif(r.lm.rem) # Testing for multicolinearity

##                                     scale(Average_cortisol, scale =
FALSE)
##                                                                1.
647698
##                                        scale(Pathogen_load, scale =
FALSE)
##                                                                2.
742447
## scale(Average_cortisol, scale = FALSE):scale(Pathogen_load, scale =
FALSE)
##                                                                2.
225720

par(mfrow = c(2, 3), oma = c(0, 0, 2, 0))
plot(r.lm.rem, ask = F, which = 1:6)
```



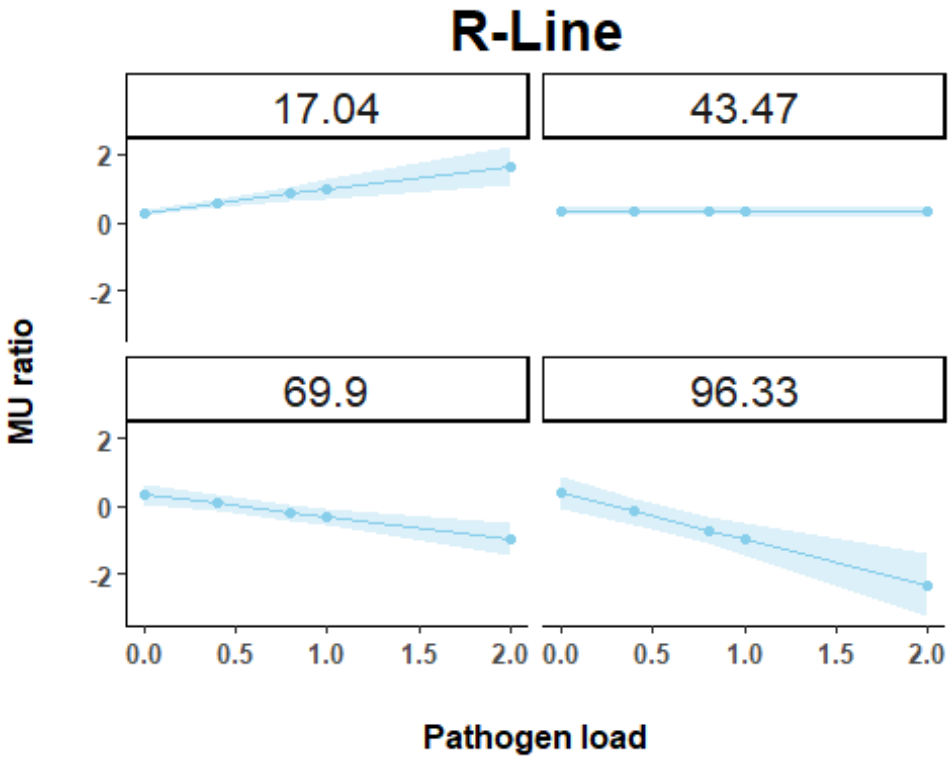

```
par(mfrow = c(2, 3), oma = c(0, 0, 2, 0))
plot(s.lm, ask = F, which = 1:6)
```



```r
# VIF - variance inflation factor - is less than 5 which tells us that
there is no issue of multicolinearity.
#All plots look okay, no visible places where assumptions are not met.

#Modeled Plots using fitted values
#S-Line
ef1 <- (effect("scale(Average_cortisol, scale = FALSE)*scale(Pathogen_l
oad, scale = FALSE)", s.lm, xlevels= list( Average_cortisol=c(17.04, 43
.47, 43.47+26.43, 43.47+(26.43*2))), multiline=TRUE, rug=FALSE))
efdata1<-as.data.frame(ef1)

hello1 <- ggplot(data=efdata1, aes(x=Pathogen_load, y=fit)) + geom_poin
t(col = "#cb4154") + geom_line(col ="#cb4154")  +
  geom_ribbon(aes(ymin=fit-se,ymax=fit+se),alpha=0.3, fill="#cb4154") +
facet_wrap(~Average_cortisol) + labs(title = "S-Line", x= "Pathogen loa
d", y="MU ratio")  + theme_classic() + theme(text=element_text(size=20)
)
Final= hello1+theme(axis.title.x=element_text(face="bold",size=12))+the
me(axis.title.y=element_text(face="bold",size=12))+theme(axis.text.x=el
ement_text(size=10,face="bold"))+theme(axis.text.y=element_text(size=10
,face="bold"))+theme(plot.title=element_text(face="bold",size=20, hjust
= 0.5))
Final_plot1 <- Final+theme(axis.title.y=element_text(margin=margin(0,20
,0,0)))+theme(axis.title.x=element_text(margin=margin(20,0,0,0)))

Final_plot1
```



```
ggsave("Final_model for serum and pathogen load in S line.png", device
= "png", width= 8, height = 6,
       Final_plot1)
#R-Line
ef2 <- (effect("scale(Average_cortisol, scale = FALSE) * scale(Pathogen
_load, scale = FALSE)", r.lm.rem,xlevels= list(Average_cortisol=c(17.04
, 43.47, 43.47+26.43, 43.47+(26.43*2))), multiline=TRUE, rug=FALSE))
efdata2<-as.data.frame(ef2)

hello2 <- ggplot(data=efdata2, aes(x=Pathogen_load, y=fit)) + geom_poin
t(col = "skyblue") + geom_line(col ="skyblue")  +
  geom_ribbon(aes(ymin=fit-se,ymax=fit+se),alpha=0.3, fill = "skyblue")
+ facet_wrap(~Average_cortisol) + labs(title = "R-Line", x= "Pathogen l
oad", y="MU ratio")  + theme_classic() + theme(text=element_text(size=2
0))
Final= hello2+theme(axis.title.x=element_text(face="bold",size=12))+the
me(axis.title.y=element_text(face="bold",size=12))+theme(axis.text.x=el
ement_text(size=10,face="bold"))+theme(axis.text.y=element_text(size=10
,face="bold"))+theme(plot.title=element_text(face="bold",size=20, hjust
= 0.5))
Final_plot2 <- Final+theme(axis.title.y=element_text(margin=margin(0,20
,0,0)))+theme(axis.title.x=element_text(margin=margin(20,0,0,0)))

Final_plot2
```



```
ggsave("Final_model for serum and pathogen load in R line.png", device
= "png", width= 8, height = 6,
       Final_plot2)
```