INNATE IMMUNE GENE EXPRESSION IN IMMUNOCOMPROMISED VERSUS IMMUNOCOMPETENT ZEBRAFISH

by

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Many bacteria live commensally in the intestines of complex host organisms, but the methods by which this community assembles are not fully understood. It is suspected that two processes, inter-host dispersal and host selection, play a role. Host selection is thought to be dictated by the immune system; however the role of the immune system in commensal host-microbe interactions is not well understood. This experiment examines the activity of the innate immune system in the context of inter-host dispersal and bacterial community assembly. To carry out this experiment, Wild-Type (WT) fish and *myd88*$^{-/-}$ fish, which were missing a key protein in the TLR pathways, were housed at different levels exposure to inter-host dispersal. I found that the immunocompromised *myd88*$^{-/-}$ fish had significantly lower levels of TLR-pathway dependent IL1B expression, but that activity of TLR-independent protein C3 was unaffected. Immunocompromised fish also demonstrated lower survival rates, and also developed less when in competition with WT fish for resources in the cohoused situation. Greater levels of host dispersal also increased the difference in activity level

between genotypes. These results suggest that innate immune activity is impacted by inter-host dispersal and by the presence of a commensal bacterial community.

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Introduction

Commensal Host-Microbe Interactions

The word "bacteria" brings to mind microscopic organisms that cause food poisoning and ear infections; harmful agents that humans have been trying to control and eradicate for years. However, though many bacteria are pathogens, billions of other species are actually not harmful at all. Recent biological research has revealed that humans, and other complex organisms, are actually host to massive numbers of microbiota in their bodies. These bacteria live commensally within the host, and form diverse and complex communities throughout the body, especially in the intestinal tract. It is gradually becoming clear that these commensal microbes are necessary for normal health and development in humans and in many other complex multicellular organisms. In humans, imbalances in this microbial community are even thought to be linked to the onset of diseases such as inflammatory bowel disease, autoimmune disorders, allergies, obesity, and anxiety[1]. The relationship between the host and these commensal bacteria is thought to be regulated by the immune system. However, though the immune system's role in host-pathogen interactions is well understood, its role in commensal bacterial-host interactions is still unclear.

Assembly of Host-Associated Microbial Communities

It is clear that the presence of this microbial community has an impact on host health and development. However, it is still not clear how these communities assemble in the first place. What is known is that host organisms are generally born without microbes and then gradually develop a microbial community by selecting from their

environment. Ecological theory can be used to predict these assembly patterns by assuming that the human microbiome acts the same way any other ecological community does[2].

According to ecological theory, one way in which bacterial communities are thought to assemble in host organisms is by the combination of two related processes[2,3]. The first is inter-host dispersal, or the idea that communities are built by transmission of microbes between host organisms. Just as pathogens are transmitted between people, the same can occur with commensal bacterial species. As demonstrated in Figure 1, the types of microbes transmitted between two adults would be different than the ones transmitted between an adult and a child, or between a healthy individual and a sick one, as demonstrated by a “healthy” black individual and a “sick red one.

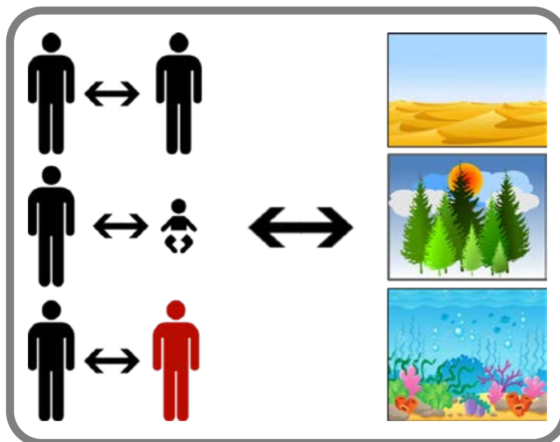


Figure 1: Inter-host dispersal and host selection in bacterial community assembly

Inter-host dispersal, shown on the left, is the process of transmitting microbes between two or more host organisms. Host-selection, shown on the left, is the environment a host provides to welcome some bacteria but to reject others. Both processes contribute to bacterial community assembly in hosts, according to Metacommunity Theory.

The second factor is host selection, or the idea that the host provides a specific environment that is more favorable for some types of bacteria and less favorable for others. In Figure 1, this is demonstrated by the juxtaposition of three different biomes that vary in the amount of water available. A seed from a forest plant could be transmit to the desert or to the coral reef via dispersal, but it would not germinate in either of the other biomes because the amount of water both other biomes is not suited to the needs

of the seed. Just as plants and animals are best suited to different habitats, the same applies to bacteria in the host intestine.

It is believed that inter-host dispersal and host selection both mediate and are mediated by each other, but the degree to which this interaction occurs is unclear.

Immune System Function

What is the factor in host organisms that selects for some bacterial species over others? Just as the selecting factor in each biome in Figure 1b was water, the selecting factor in host organisms is thought to be the immune system. This was mentioned earlier and is what we set out to investigate with our experiment.

The immune system is necessary for most multicellular organisms to maintain health. It provides protection against pathogens by distinguishing self, or host cells, from non-self, or any other foreign particle.

There are two branches in the immune system: the innate immune system and the adaptive immune system. The innate immune system is a generalized defense mechanism that is conserved in all animals and recognizes markers common to many microbial species but absent in animals. In contrast, the adaptive immune system is only present in vertebrates, and provides specific defense against foreign particles encountered during hosts' lifetimes[4].

The immune system's role in host-pathogen interactions is well understood. However, much about its role in commensal host-microbe interactions is unclear, because the very presence of a commensal microbial community in hosts suggests that the immune system is not only able to distinguish between self and non-self, but also

can go so far as to distinguish microorganisms that are beneficial versus ones that are harmful to the host.

The MyD88 pathway

One innate immune pathway of interest in commensal host-microbe interactions is the Toll-Like Receptor (TLR) pathway. It is highly conserved between many species, and is a central pathway in the innate immune system[5,6]. In addition to its role in defending against many common pathogens, it has also been shown to play a role in the regulation of commensal baceteria[7].

The TLR pathway is a signal transduction pathway. The pathway is activated when a pathogen, or a non-self particle, is recognized by toll-like receptors on the outside of intestinal immune cells. The receptors then activate the Myeloid Differentiation Primary Response Gene 88 (MyD88) protein inside the cell, which then activates many other proteins[6]. Eventually, the message is passed down to the cell nucleus, where the cell is directed to produce and release proteins to defend against the pathogen. One class of defensive proteins is pro-inflammatory cytokines, which are small proteins that cause inflammation in order to attract other immune cells to the affected area[8]. If large amounts of bacteria are present in the host, then the innate immune system will produce large quantities of cytokines. Eventually, this response will kill the bacteria that first activated the TLR pathway.

However, recent studies have shown that the TLR pathway is not only important in defending against pathogens, but also regulated commensal bacteria populations[7]. When the bacterial product Lipopolysaccharide (LPS) is present in the host, then the TLR pathway activates the production of Intestinal Alkaline Phosphatase (IAP) along

with pro-inflammatory cytokines. IAP then detoxifies LPS from commensal bacteria, which prevents the immune system from activating a pathogen-associated septic shock response. This allows hosts to regulate and maintain a healthy commensal bacteria community[7].

While there is a strong understanding of how the TLR pathway changes in the presence of commensal bacteria, much is still not understood about the actual interaction between this pathway and commensal bacteria. For example, it is not clear how large of a role the TLR pathway plays in selecting for bacteria in healthy hosts.

One way to learn more about the role of the TLR pathway's role in bacterial community assembly is to study genetically modified host organisms. Take, for example, an organism in which the intermediary protein MyD88 is missing. Both the healthy hosts and immunocompromised hosts will still accumulate an intestinal microbial community, but some bacteria species may flourish in immunocompromised hosts that would not be able to grow in the healthy hosts. Looking at the difference in immune activity between different hosts with differing microbiota could provide valuable insight into how the innate immune system is impacted by loss of an important pathway, and by alterations in the intestinal microbial community.

Specific Aims

The main goal of this experiment was to characterize differences in innate immune activity among hosts, and how those differences might interact with the microbiota and inter-host dispersal. This was specifically carried out by looking at two ecological processes; inter-host dispersal and host selection, specifically the role of the

immune system. In order to address this goal, we divided it into three specific aims that target each part of the question.

1. How does the absence of the TLR pathway influence the overall innate immune response in healthy fish? On a basic level, much about the interaction between commensal bacteria and the immune system is not known, and it is also unknown how hosts will respond to the microbiota when they lack a key pathway in their innate immune system.
2. How does inter-host dispersal influence host innate immune activity? This question will help address the relationship between inter-host dispersal and host selection, in terms of the role both forces play in bacterial community assembly, and what the consequences of bacterial transmission on hosts are.

Hypotheses

For my first specific aim, I hypothesize that the absence of the TLR pathway will negatively impact host health and the overall strength of their immune response, even though the samples are not actively being exposed to pathogens. I expect this because the immune system plays a role in the maintenance of health, not just in defense against pathogens, and because previous literature demonstrates that the TLR pathway does play a role in maintaining commensal bacterial communities[7]. However, while I expect the absence of the TLR pathway to impact TLR-dependent immune responses, I don't expect other pathways in the innate immune system, such as the complement pathway, to be affected.

For the second specific aim, I hypothesize that immunocompromised fish will have lower innate immune activity regardless of the degree of inter-host dispersal, as they are missing part of a crucial immune pathway.

Furthermore, since increasing inter-host dispersal will likely increase the exposure of hosts to a wider diversity of bacteria, I expect that when fish are housed by genotype (WT hosts with WT hosts, immunocompromised hosts with immunocompromised) then innate immune activity will differ greatly between genotypes, but that within genotypes innate immune activity will be pretty constant. Between WT hosts cohoused with immunocompromised hosts, I expect innate immune expression to be highest in both genotypes.

Model Organism

The model organism used in this experiment was the zebrafish (*Danio rerio*). Zebrafish were first established as a model organism in 1981 by George Streisinger, a University of Oregon professor[9]. Streisinger wanted to develop an organism that could be genetically manipulated and that was a good model for human diseases, and zebrafish were found to suit both those needs. They were used as a model in developmental biology for many years, but in the last two decades have also been used to study immunology and the impact of infectious diseases[10], and as pathogenic host-bacterial interactions have been studied in zebrafish, it became ideal to also use this well-established model to study commensal host-bacterial interactions.

Because of how well-established zebrafish are in the study of commensal host-microbe interactions, they are ideal model in this experiment. Unlike mice, they lay large clutches, which make it possible to generate large, statistically significant sets of

data, and can also be easily raised in a variety of conditions, making it easy to control inter-host dispersal between samples[11]. Well-established genetic manipulation techniques in the zebrafish model make it possible to modify immune activity in the fish, which can be used to control host selection. Finally, many methods exist to control and manipulate the microbial community within these fish, such as the capacity to make them totally free of bacteria, or "germ-free"[12].

Experimental design

In order to determine how host-selection and inter-host dispersal impacted the development of the innate immune system in zebrafish, each mechanism was isolated in different experimental conditions. To test host selection via innate immunity, two different zebrafish genotypes were used. Wild-type (WT) zebrafish were fish with a normal genome, that had normal levels of immune activity. MyD88 CRIPSr mutants (*myd88*$^{-/-}$) were a genetically engineered line of fish missing the MyD88 protein, the adaptor for the TLR pathway described earlier, and hence their innate immune activity is compromised[13]. To test the role of inter-host dispersal in innate immune development, samples from each genotype were raised in three different housing treatments. In the first treatment, fish from both genotypes were kept in individual containers, so no inter-host dispersal was possible. In the second treatment, fish of the same genotypes were housed together, but the genotypes were kept separated so that inter-host dispersal was limited to only fish of the same genotype. In the final environment, fish from both genotypes were housed together, so that dispersal among hosts of both genotypes was possible. The amount of water per tank and amount of food given in each treatment was scaled according to number of samples per flask, so that

oxygen and nutrient distribution was kept constant across treatments. A diagram of the experiment is depicted in Figure 3.

The fish eggs were treated to remove all bacteria after embryogenesis, ensuring that no outside microbes influenced the trials[12]. After this, samples were distributed into the given treatments and were raised in the different housing conditions for 21 days. Since adaptive immunity develops between 21 to 28 days post fertilization in zebrafish, raising the fish to 21 days ensured that the effects of innate immunity were isolated. Upon completion of the trial, all samples were analyzed for expression of cytokines that were both dependent and independent of the MyD88 pathway.

The MyD88-independent cytokine tested was complement protein 3 (C3), a protein that is key to the complement pathway. The complement pathway is a different innate immune pathway used in defense against bacterial pathogens, and while several different pathways are present in zebrafish, all of them converge at C3 cleavage, which is used to promote inflammation and to activate other defense mechanims[14]. The MyD88-dependent cytokine was interleukin-1β (Il-1B), a pro-inflammatory cytokine that is activated by TLR via the MyD88 protein[7].

Both Il-1B and C3 were chosen because of their central role in each innate immune pathway being observed, so the relative levels of expression of each cytokine are also an indication of the overall level of activity in each pathway. The MyD88-dependent cytokine was also chosen to verify that the mutant fish line was demonstrating knockout of the *myd88* gene.

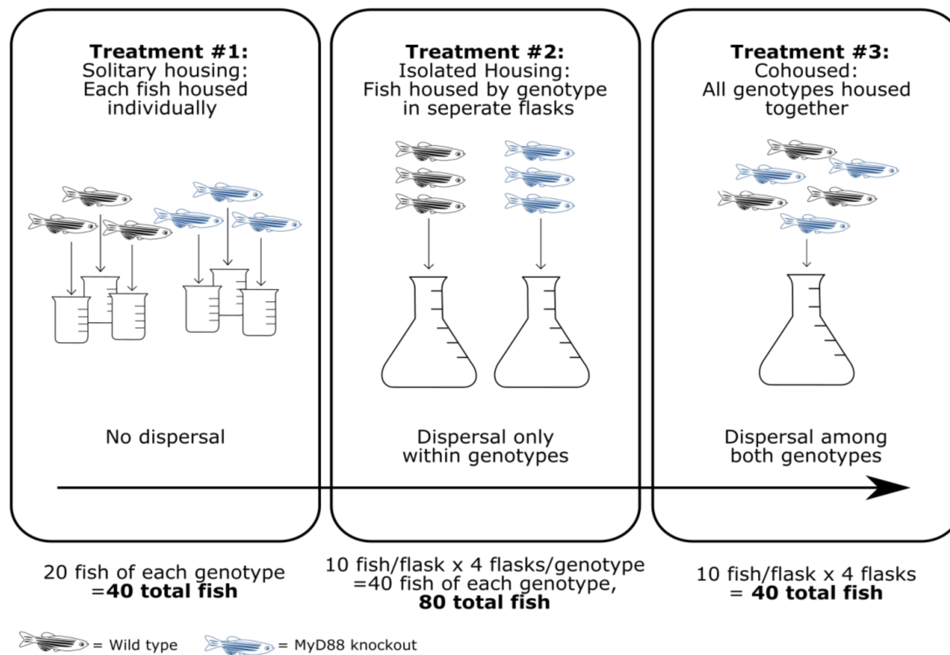


Figure 2: Experimental Design

Six total treatments characterize all combinations of host selection and dispersal. Treatment #1: One fish per container, 20 containers containing Wild-Type (WT), 15 containing MyD88 knockout (*myd88*$^{-/-}$) samples. Treatment #2: 10 WT fish in one container, 10 *myd88*$^{-/-}$ fish in another container, 3 replicates of each container. Treatment #3: 10 fish, both WT and *myd88*$^{-/-}$, housed together in one container, 3 replicates of each container. Total 120 samples prepared, 60 WT, 60 *myd88*$^{-/-}$.

Analysis techniques and the Central Dogma of Biochemistry

At the completion of the treatment, fish lengths were measured for each sample in every treatment. Length is a fairly accurate indicator of overall zebrafish development, and was used to determine how each housing treatment impacted overall fish health[15].

Analysis of innate immune cytokine levels was completed by quantitative-Polymerase Chain Reaction (qPCR), which is a process that measures the amount of RNA transcripts produced for each cytokine.

This method of analysis is based on the central dogma of molecular biology, which is that DNA encodes RNA, and that RNA encodes protein. DNA or, Deoxyribonucleic Acid, is an inheritable macromolecule that contains the biological information necessary to life. DNA consists of two long strands that wrap around each other to form a double helix. Each strand is made up of smaller molecules called nucleotides, which form specific sequences and run complementary and anti-parallel to each other.

Only one copy of DNA is present in the cell and it is stored in the nucleus, which protects it from damage. To get information from DNA out into the extracellular matrix, where it will be utilized, a molecule called messenger ribonucleic acid, or mRNA is synthesized and transported out of the nucleus in a process called transcription. RNA has many similar properties to DNA, except that it is single stranded. When information from DNA needs to be transmitted to the rest of the cell, the relevant portion of the helix unwinds, and a complementary mRNA strand is built upon the exposed DNA strand. Once completed, the mRNA strand detaches from the DNA and leaves the nucleus. Unlike DNA, mRNA is synthesized in proportion to the amount of material eventually needed in the cell. So, for example, all cells in the human body contain the same DNA, but the types of mRNA synthesized in a muscle cell will be different from the ones synthesized in a liver cell, not only in which transcripts are used, but in the number of mRNA copies made from the original DNA.

Once mRNA exits the cell, it then is captured by cell organelles called ribosomes, which use the mRNA to synthesize proteins in a process called translation. Ribosomes recognize three-nucleotide-long sequences on mRNA, called codons. There

are 64 possible codons, which correspond to 20 types of transfer RNA (tRNA). Each tRNA molecule has an anti-codon that is complementary to codons on one side, and is attached to a specific amino acid on the other side. As each codon is matched to a tRNA anti-codon, the amino acids join together via peptide bonds, and create a polypeptide. Once the mRNA sequence ends, the protein is released from the ribosome and rapidly folds into a specific shape to form a protein.

Proteins are used for every biological process. They provide structure and shape to cells, transfer nutrients, catalyze metabolic reactions, respond to stimuli, regulate the amount of DNA replication and RNA synthesis that takes place in a cell, and help the cell replicate and reproduce. Every function a living organism needs to carry out is done by proteins.

qPCR is a process in which cDNA transcripts are amplified exponentially, and the quantity of DNA in the reaction mixture after each round of amplification is measured via a fluorescent dye. To perform this procedure, I converted RNA to cDNA. The number of RNA transcripts in the cell is proportional to the activity of the protein, which allows us to use this measure to determine the activity of different immune cell proteins. Distinct proteins are very difficult to extract because of their complex three-dimensional structure, but DNA and RNA transcripts can be easily extracted and measured because of chemical properties of the molecule and because of their specific sequences. Complementary sequence fragments can be used to bind RNA fragments.

Experimental Procedures:

Zebrafish Husbandry

All zebrafish experiments were performed following protocols approved by the University of Oregon Institutional Animal Care and Use Committee. Germ-free embryos, or embryos sterilized so that no bacteria were present on them, were produced following standard protocol[16]. Isolated and Cohoused fish were kept with 10 fish per flask, and solitary samples kept in individual flasks. Water (E6 media) and food was distributed in proportion to the number of fish per flask, with 50 mL of water per fish.

Zebrafish sampling and RNA Extraction:

All fish samples were sacrificed using tricaine methane sulfonate (MS222, Sigma), and measured their standard length. The intestines were removed for use in a related analysis of intestinal microbial communities, and the carcasses were stored in Trizol at -80° C (Invitrogen). RNA was harvested by homogenizing the samples and extracting the organic solvent using the Trizol reagent. The extract was further purified with a GeneJet RNA Purification Kit (ThermoFisher).

Enumeration of Innate Immune Activity using Real-Time Quantitative (q)RT-PCR

The RNAs were used as templates for generating coding single-strand DNAs (cDNA's) with Superscript IV reverse transcriptase and random primers (Invitrogen) following the manufacturer's instructions. After that, cDNA's were purified with GeneJet PCR purification kit (ThermoFisher). qRT-PCR assays were performed in 20 ul reactions with cDNA corresponding to 20 ng of cDNA, and 400 nM gene-specific or

control primers. Gene-specific primers were ordered from Eurofins Genomics. Primer sequences are as follows; IL-1B: F: 5'CATCAAACCCCA ATCCACAG-3', R: 5'-CACCACGTTCACTTCACGCT-3'; C3: F: 5'-CGGACGCTG ACATCTACCAA-3', R: 5'-TCCAGGTCTGCTCT CCCAAG-3'. Housekeeping genes SDHA and ElF-1B primers used to normalize the results were ordered from PrimerDesign. All reactions were performed in Bio-Rad CFX96 Real-Time PCR (qPCR) Thermocycler.

Statistical Analysis

qPCR results were normalized using LinReg analysis software. Variation in cytokine expression across genotype was performed using two-sample t tests assuming unequal variances in RStudio. Pearson Correlations assuming linear relationships between variables were conducted between both innate immune proteins, and also between each protein and standard length. Both statistical methods were then used to analyze housing and genotype effects on expression.

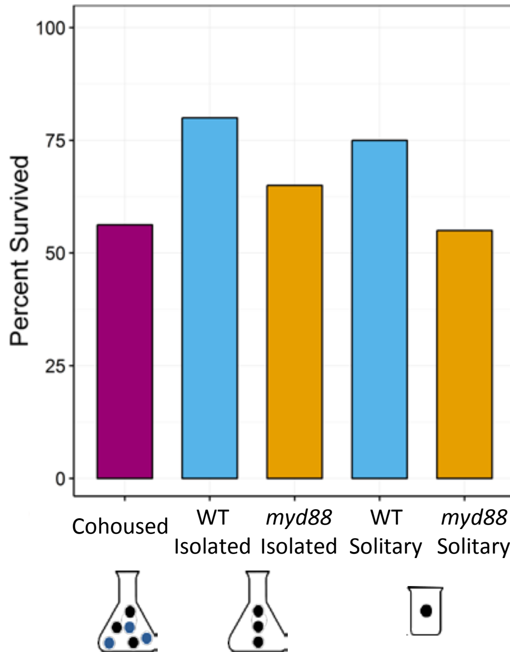
Results:

Influence of the MyD88 protein on Fish Survival

Survival was measured by counting how many fish in each treatment were still alive after 21 days post fertilization. The percentage of survivors per treatment is compared below in Figure 3. Additionally, Table 1 demonstrates how many samples survived each treatment.

Among WT samples, 80% of the fish in the solitary treatment survived, and 75% in the isolated treatment. Since the survival rate of all lab-reared WT zebrafish is about 80%, these rates demonstrate that the samples were not subjected to undue stress or intolerable living conditions during the experiment. Despite this, the *myd88* mutants had much lower survival rates. Only 65% of the solitary mutants survived, and only 55% of the isolated ones, so on average about 22.7% fewer mutant fish survived both treatments. This demonstrates that even unchallenged fish seemed to suffer from being immunocompromised.

In the cohoused circumstance, only 56.75% of fish from both treatments survived, which suggests that both genotype experienced larger die-offs in this treatment. This is especially true for the WT fish, which had relatively high survival rates in the other treatments, however the *myd88*$^{-/-}$ samples probably had similar or only slightly lower survival rates. Cohousing both genotypes together appears to be detrimental to the health of both genotypes, but especially the wild-type genotype.



Treatment	First Count	Final count	Percent survived (%)
Solitary, WT	20	16	80
Solitary, *myd88*	20	13	65
ted, WT	40	30	75
Isolated, *myd88*	40	22	55
Cohoused,	40	27	56.25

Table 1: Percentage of zebrafish in each treatment that survived 21dpf

Figure 3: Zebrafish survival rates across treatment

Percentage of fish in each treatment that survived to 21 days post fertilization. Purple represents the cohoused treatment with mixed WT and *Myd88*$^{-/-}$ genotypes, blue represents the WT samples in the Isolated and solitary samples, and yellow represents the *myd88*$^{-/-}$ in the isolated and solitary treatments.

Characterization of Immune Activity in the absence of *myd88*

Closer inspection of immune activity via analysis of C3 and IL1B relative expression levels demonstrates that *myd88*$^{-/-}$ mutants have significantly lower expression of IL1B. As shown in Figure 4a, expression of IL1B is much lower in *myd88*$^{-/-}$ mutants. On the other hand, expression of C3 (shown in figure 4b) appears to be higher in *myd88*$^{-/-}$ samples, but the large standard error means that this difference is not significant, demonstrating that *myd88*-independent immune pathways are unaffected by loss of *myd88*.

Figure 4c and 4d correlate activity of IL1B and C3 in wild-type and mutant fish regardless of housing. In the WT samples, a positive correlation is present, demonstrating that when C3 activity is high, IL-1B activity is higher as well. This

confirms that the WT samples had normal innate immune activity, since any health risk is equally activating both pathways.

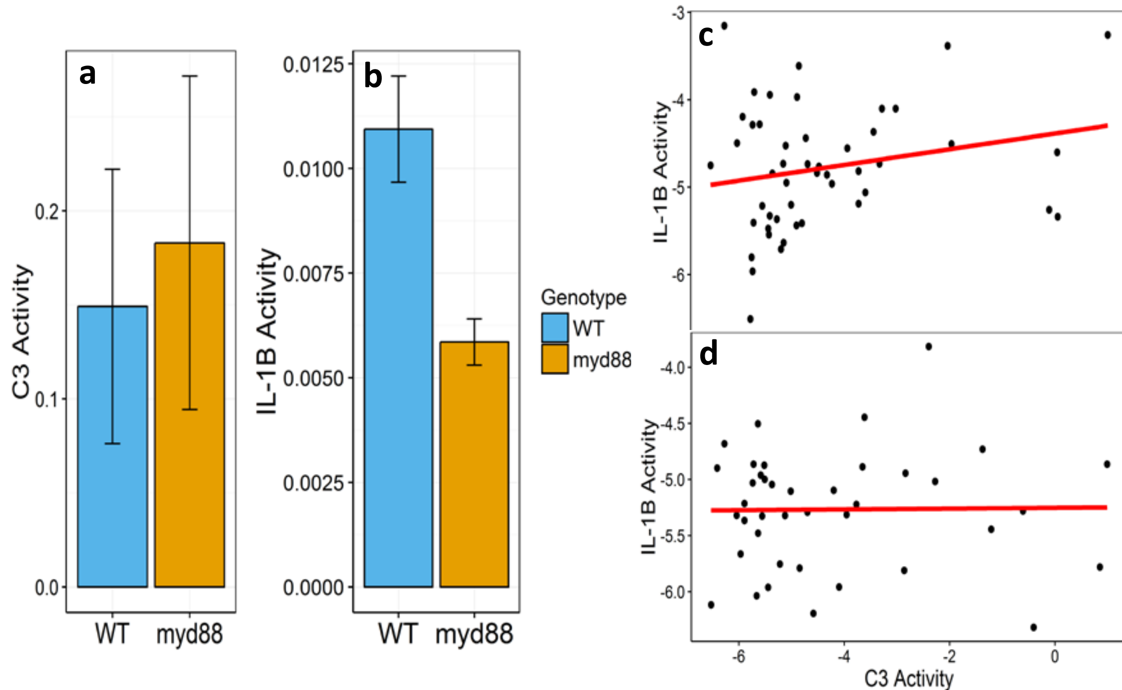


Figure 4: C3 and IL1B activity in WT and *myd88*$^{-/-}$

Average C3 activity does not vary by genotype (a). Though the average appears higher in *myd88* knockouts, the p-value equals 0.77, and the difference is not significant. Average IL1B activity, however, is significantly different between genotypes (p = 0.0048), and activity is much lower in the *myd88* mutants (b). In WT samples, there is a positive correlation between IL1B and C3 expression (c). The correlation equals 0.35, and has a p-value of 0.016. In *myd88* knockout fish, the correlation between C3 and IL1b activity equals -0.04, and p = 0.80 (d), demonstrating that no significant correlation is present.

However, in the *myd88*$^{-/-}$ fish, this correlation is no longer present. No matter how high C3 activity gets, IL1B activity remains constant. This demonstrates that IL1B is greatly depleted in the *myd88*$^{-/-}$ fish, resulting in an incomplete immune response across a range of immunostimulation.

Influence of Housing and Immune Activity on Fish Development

Despite lower survival rates among both genotypes when cohoused, WT samples seemed to develop best when exposed to higher levels of inter-host dispersal. As shown in Figure 5, there is a statistically significant difference in average standard length between the WT solitary and WT cohoused samples, with cohoused fish being on average 12% larger than solitary samples. This correlation, however, is absent in the *myd88*$^{-/-}$ samples, and no significant differences are found in fish length across all treatments. This suggests that immune deficiency in the *myd88*$^{-/-}$ samples is overwhelming the beneficial effects of cohousing.

In comparing across genotype, the *myd88*$^{-/-}$ fish actually are larger than the WT fish in the solitary and isolated housing treatments, though this difference is not significant. However, when *mys88*$^{-/-}$ and WT fish are housed together, then the *myd88*$^{-/-}$

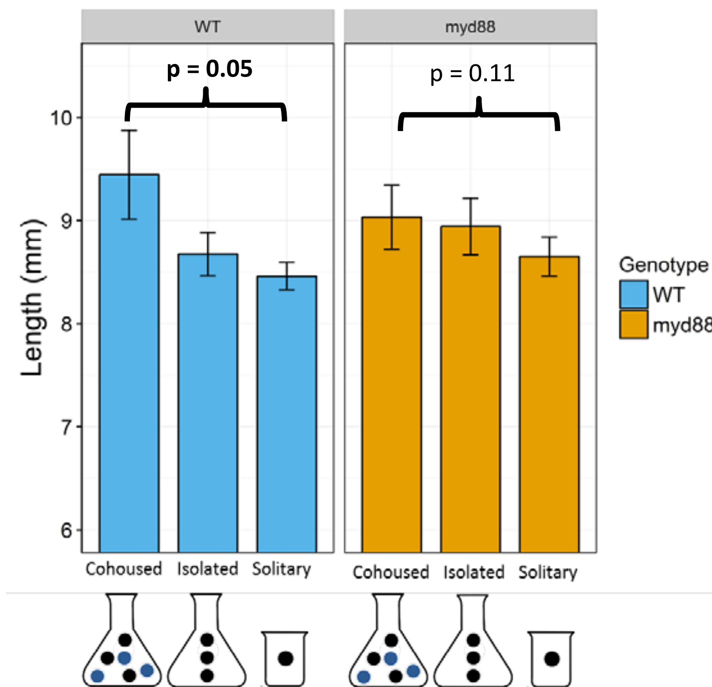


Figure 5: Standard length across treatments

Average standard length across genotype and treatments shown by bars, and standard error is denoted by the error bars. P-values between cohoused and solitary treatments for WT and *myd88*$^{-/-}$ samples are shown above bars. The difference between cohoused and solitary WT fish is statistically significant, but *myd88*$^{-/-}$ samples do not demonstrate the same relationship.

are on average 0.4 mm shorter than the WT fish. This difference could be because the *myd88*$^{-/-}$ fish are less able to compete against WT fish for food and resources.

Standard length can also be correlated to innate immune activity, in order to determine the relationship between health and development. As shown in Figure 6, the WT/IL1B correlation is the only one that generates a statistically significant relationship, and the correlation is negative. This demonstrates that WT fish with more IL1B activity are less developed, possibly because they are directing more energy towards activating the TLR pathway to fight pathogenic bacteria, and hence do not have the energy to develop sufficiently.

The *myd88*$^{-/-}$ fish also demonstrate a negative correlation between IL1B expression and length, but this correlation is very small and is not statistically significant, demonstrating that IL-1B activity is lowered or impaired in the mutant fish.

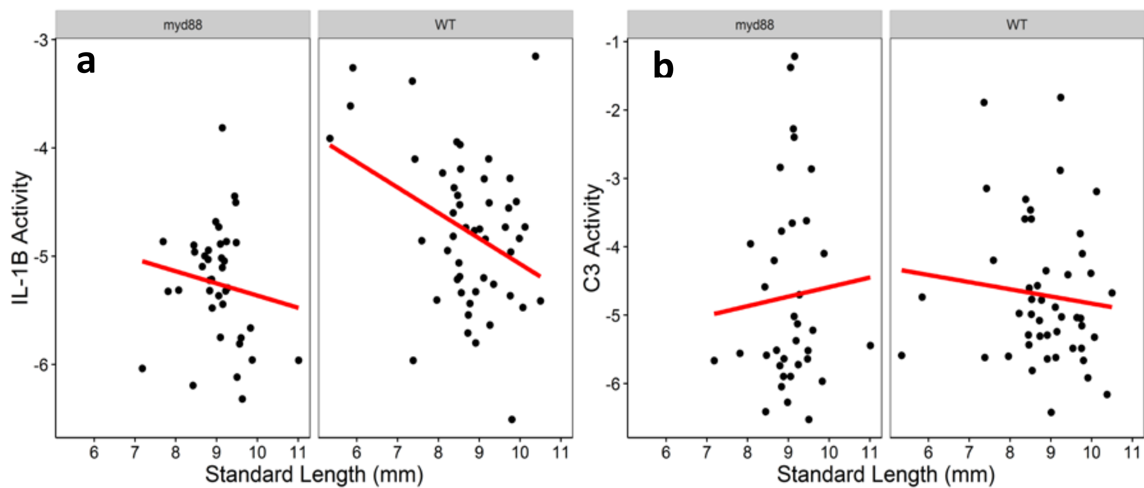


Figure 6: Correlation between IL1B/C3 activity and Standard Length

IL1B activity and standard length are correlated in WT fish: p = 0.016, Correlation = -0. 35. However, IL1B and standard length are not significantly correlated in *myd88* mutants: p =0.80, Correlation = -0.42 (a). C3 activity and standard length are not significantly correlated in either genotype. In WT fish: p = = 0.467, Correlation = -0.19. IN *Myd88* mutants, p = 0.7812, Correlation = 0.049

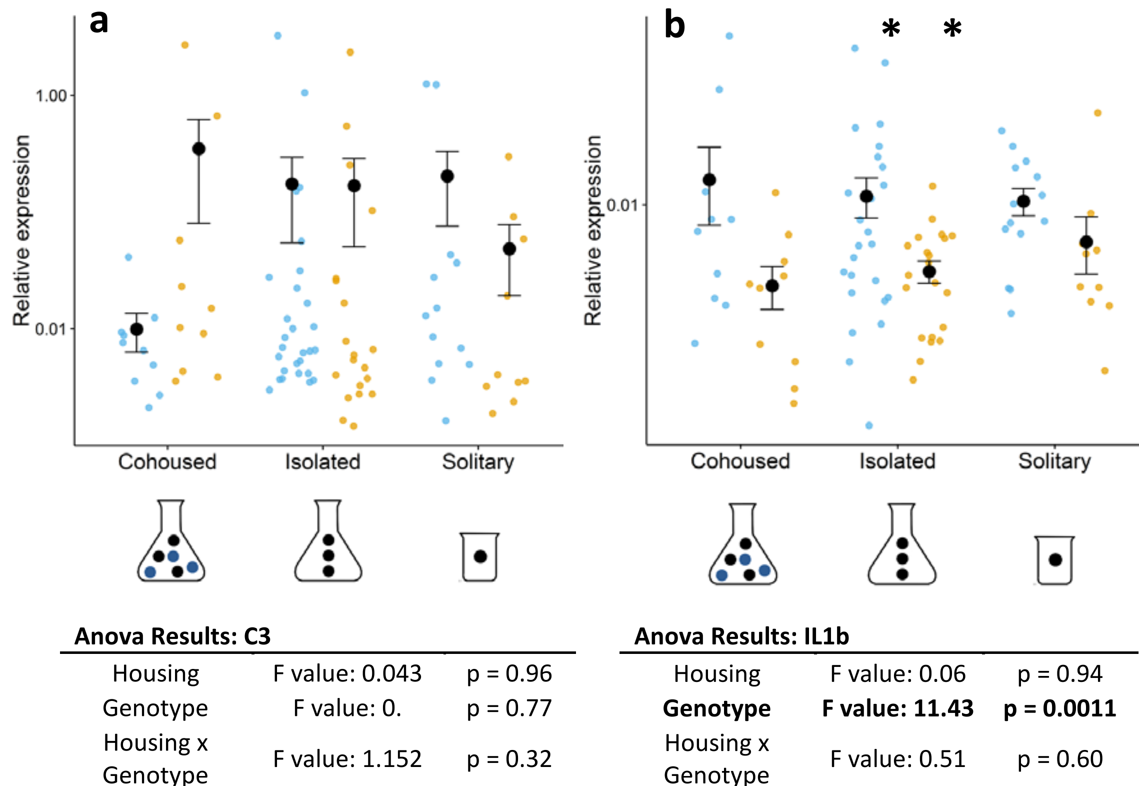
The correlation between C3 activity and fish length is not significant for either WT or mutant fish. This is unexpected because, as in the WT/IL1b correlation, it would be expected higher levels of innate immune activity would occur in less developed fish. However, the lack of correlation could be because the complement pathway is a different mechanism that doesn't affect development the way the TLR pathways does.

C3 and IL1B expression by treatment

Average expression for C3 is unchanged across genotype and across housing treatments, as shown in Figure 7a and Table 2. The one exception appears to be between WT and *myd88*$^{-/-}$ fish, but even this difference is not significant, probably due to high standard error, suggesting increased sampling may be necessary to detect differences.

On the other hand, IL1B expression does vary between housing treatments. As shown in figure 7b, there is variation across genotype in all treatments. Table 3 shows results of the ANOVA test, and demonstrates that $p = 0.0011$ between genotypes across all treatments. Furthermore, it appears that the greater the inter-host dispersal, the more divergent IL1B activity is. In the totally solitary treatments, both WT and *myd88*$^{-/-}$ samples have relatively similar levels of IL1B expression, and there is a relatively large standard error across the my88$^{-/-}$ samples. The isolated samples are the only ones that demonstrate significant difference across genotype in a pairwise t-test, and have very low variance. Finally, the cohoused samples have the greatest difference between genotypes. The *myd88*$^{-/-}$ cohoused samples have the lowest IL1B expression out of all the *myd88*$^{-/-}$ housing treatments, and the WT cohoused samples have the highest IL1B

expression out of all the WT housing treatments. However, the variation is slightly less than significant, probably because of large standard error in the WT samples.



Anova Results: C3

Housing	F value: 0.043	p = 0.96
Genotype	F value: 0.	p = 0.77
Housing x Genotype	F value: 1.152	p = 0.32

Anova Results: IL1b

Housing	F value: 0.06	p = 0.94
Genotype	**F value: 11.43**	**p = 0.0011**
Housing x Genotype	F value: 0.51	p = 0.60

Figure 7: Variation in C3 and IL1B activity across housing treatments.

Expression of C3 (a) and IL1B (b) across housing treatments. Blue represents WT fish, yellow represents *myd88* knockouts. For C3, p = 0.24 between WT and *myd88* knockout samples in the cohoused treatment; p = 0.97 between isolated samples; p = 0.25 across solitary samples. For IL1B, p = 0.10 between WT and *myd88* knockouts in cohoused samples; p = 0.012 between isolated samples (represented by stars in (b)); p = 0.17 across solitary samples.

Table 2: ANOVA results for C3across housing and genotype

Table 3: ANOVA results for IL1B across housing and genotype

Statistically significant variation across multiple groups is shown in bold.

Discussion

As hypothesized in the first specific aim, *myd88* has a strong influence on fish survival and on healthy immune system activity, even in unchallenged or uninfected fish. Fish samples that are missing *myd88* have a limited immune response, but still maintain normal levels of activity in other pathways, such as the complement pathway. This confirms that *myd88* has an important role in innate immunity, but is not the only significant pathway in the innate immune system.

While C3 does not show significantly differences across genotype or housing treatment, IL1B activity is significantly lower in *myd88*$^{-/-}$ fish. In the solitary housing treatments, *myd88*$^{-/-}$ had high standard error, as expected in the solitary housing, but WT samples do not have as high of error. If the bacterial communities were characterized in the solitary samples, then I expect that the WT fish would all have similar communities, but that the *myd88*$^{-/-}$ fish will have large variation in the community makeup.

In the isolated housing treatment, the WT and *myd88*$^{-/-}$ samples were very different from each other, and both treatments had very little variance in IL1B activity. This was as expected in my hypothesis: inter-host dispersal caused all the fish in each treatment to encounter a very similar cohort of bacteria, causing the same host-selection processes to occur within each genotype. So, each genotype was very similar in IL1B activity, but the genotypes differed due to different host selection processes. I suspect that if the bacterial communities were characterized in these samples, then the WT fish and *myd88*$^{-/-}$ fish will have very distinct communities. The WT isolated fish bacterial community would match that of the WT solitary samples, but the *myd88*$^{-/-}$ isolated community would probably be different from that of the *myd88*$^{-/-}$ solitary samples.

In the cohoused treatment, the WT samples had high standard error and higher levels of IL1B activity, and the *myd88* samples had some standard error and somewhat lower IL1B activity than in other treatments. This suggests that cohousing the fish, and allowing inter-host dispersal across both genotypes, overwhelmed host selection processes. A likely possible explanation is that *myd88*$^{-/-}$ samples transmit bacteria to the WT samples that the WT immune system would normally reject. The increased presence of these bacterial species must have activated the TLR pathway to a greater extent than happened in the isolated and solitary WT fish, because the main means by which these bacteria were transmitted was via the immunocompromised *myd88*$^{-/-}$ fish.

On the other hand, the *myd88*$^{-/-}$ samples were probably exposed to more bacteria from the WT fish that did not activate IL1b, causing relatively lower expression of it in the *myd88*$^{-/-}$ fish. I suspect the bacterial communities in the cohoused WT and *myd88*$^{-/-}$ ish are very similar, but that this community will consist of a mix of different bacterial species from both genotypes.

The presence of “more pathogenic” bacteria in the intestines of WT cohoused fish could also explain why cohoused WT fish had a relatively lower survival rate, but still had high development. Since Figure 6 demonstrated that smaller WT fish had higher levels of IL1B expression, it could be that some WT cohoused fish had a more negative response to bacteria transmitted from the *myd88*$^{-/-}$ samples. This could have caused smaller WT fish in the cohoused treatment to die off. This would have only left larger, better developed WT in the cohoused treatment that were strong competitors for resources, and were better able to handle the bacteria transmitted from *myd88*$^{-/-}$ samples.

It would be very useful to look at the bacterial community characterization in order to determine whether these assumptions are upheld. Additionally, it would be interesting to manipulate adaptive immune response in future experiments to see how host-selection processes differ after the onset of adaptive immunity. Finally, from an ecological theory perspective, it would be interesting to see how altering inter-host dispersal impacts the transmission of pathogenic bacteria between different genotypes. The experimental framework and results discussed here could be of significant assistance in interpreting the results of these future studies.

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