Leveraging the Transparent Zebrafish to Test for Conserved Gene Function Between Mammals and Fish

Lena Stasiak

Follow this and additional works at: https://digitalcommons.library.umaine.edu/honors

Part of the Animal Sciences Commons, and the Biology Commons

This Honors Thesis is brought to you for free and open access by DigitalCommons@UMaine. It has been accepted for inclusion in Honors College by an authorized administrator of DigitalCommons@UMaine. For more information, please contact um.librarytechnical.services@maine.edu.
LEVERAGING THE TRANSPARENT ZEBRAFISH TO TEST FOR CONSERVED
GENE FUNCTION BETWEEN MAMMALS AND FISH

by

Lena Stasiak

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Zoology)

The Honors College
University of Maine
May 2020

Advisory Committee:
Robert Wheeler, Associate Professor of Microbiology, Co-Advisor
Clarissa Henry, Associate Professor of Biological Sciences, Co-Advisor
Mimi Killinger, Rezendes Preceptor for the Arts in the Honors College
Melody Neely, Associate Professor of Molecular and Biomedical Sciences
Jared Talbot, Assistant Professor in the School of Biology and Ecology
ABSTRACT

Throughout the United States and Europe, *Candida albicans* remains the most clinically significant fungus. While fungi account for thousands of deaths annually, the most clinically significant bloodstream infection in ICU patients, causing as high as 50% mortality, is disseminated candidemia. This systemic infection occurs when the normally commensal and harmless fungi, *Candida albicans*, becomes pathogenic in immunocompromised individuals. Many factors of the immune system can be paralleled between humans and zebrafish, which can be a useful model host to understand the cellular immune responses to infection. One particular gene known as myeloid differentiation factor 88 (*myd88*) has been previously found to be an important recruiter of immune cells in the innate immune response to infection. Paradoxically, previous work on mucosal infection in the zebrafish swimbladder did not show impaired macrophage recruitment between *myd88* mutant fish and wild type fish, as was expected. In this Honors Thesis study, we aimed to investigate if this disconnect was due to *myd88* playing a different role in mucosal vs. the disseminated infection models. *myd88* was shown to protect against hindbrain infection. Additionally, we found that the NF-κB pathway was activated during a hindbrain infection, and appears to act independently of *myd88*. This may provide insight into the selective role *myd88* plays in immunity to *C. albicans* in a localized hindbrain infection.
ACKNOWLEDGEMENTS

I would like to thank all researchers in the Wheeler Lab for their helpfulness and kindness. I would especially like to thank Bailey Blair who trained me to be able to do all methods that this project required, for answering endless questions, and for never hesitating to help in any way possible. I would not have been able to even start this process without her guidance.

I would like to thank my family and friends who have had my back throughout this year and who have provided me with the motivation I needed to succeed.

I would like to thank Mark Nilan at the UMaine Zebrafish Facility for providing care to the university’s zebrafish.

I would like to thank the members of my Thesis Committee. Thank you to Dr. Neely and Dr. Talbot for becoming apart of this project without having known me beforehand, and for trusting in my ability to succeed. Thank you to Dr. Killinger for your extensive help with my reading list, and for being such a friendly face and comforting presence. Beyond this project, thank you to Dr. Henry and Elisabeth Kilroy for initially providing me the opportunity to work in a research lab. The time I spent in the Henry Lab allowed me to develop skills as an undergraduate researcher, and was also a lot of fun. Because of that experience I have changed my future career path, I truly do not know what I would be doing now had I not gotten as lucky as I was to have that opportunity.

Finally, thank you so much to Dr. Wheeler. The amount of time you have spent helping me with this project from weekly meetings to edits on my writings and presentations has not gone unnoticed or unappreciated. I have learned so much through your extensive
knowledge and the ability you have to communicate it in ways that are comprehensive to me. Thank you for always being approachable and for encouraging me along the way!

Research reported in this project was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103423. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.
FOREWORD

By working on this project I have learned a lot beyond the inner workings of the innate immune system and the techniques that made this research possible (spawning, injections, screening, imaging, etc.) I have also realized how frustrating and difficult it is to produce reliable and replicable data. There are so many aspects that go into planning for a research project, and even more that pop up along the way. The entire premise of this project was changed many times due to limitations such as fish availability, the time I had to complete the project, and my weekly schedule which changed from the fall semester to the spring. I definitely embarked on this research with specific goals in mind for myself and for the outcome of my data that were unrealistic in the context of a doable project for an undergraduate, and also ignoring the fact that I may not have any data at all. A lot of the research process is about trial and error, learning and building off of mistakes along the way, and even changing directions with the question initially being set out to answer. It has not been exactly easy to accept all of the obstacles that appeared over this past year in my attempt to complete this project. I definitely became a bit consumed by the idea of an end goal and an answer, when really no research in this field is ever complete. There will always be more to discover and expand upon in the microbiology and immunology realm, which upon reflection, is a fact to celebrate rather than to feel defeated by. All and all, I am proud of the time and effort that I put into this project, I am so thankful to numerous people who have helped me along the way, and I am ever more looking forward to a future in research of equal parts frustration and fascination.
TABLE OF CONTENTS

INTRODUCTION ........................................................................................................... 1
  Overview of fungal infection, specific to *Candida spp.* - *C. albicans* ................. 1
  Overview of the Innate Immune System and *C. albicans* evasion mechanisms ........................................................................................................................................... 3
   *myd88* Dependent Pathways Including NF-κB and IL-1 ........................................ 6
  Cytokines and TNF-α ................................................................................................ 7
  Overview of Project .................................................................................................. 8
  Zebrafish as a Model ............................................................................................... 12
  Significance of Project ........................................................................................... 13

MATERIALS AND METHODS ...................................................................................... 14
  Zebrafish Care and Maintenance ......................................................................... 14
  Candida Growth Conditions and Preparation ...................................................... 16
  Needle Preparation and Hindbrain Microinjection ............................................... 16
  Vivatome Screening ............................................................................................... 18
  Confocal Microscopy ............................................................................................. 18
  Statistics .................................................................................................................. 20

RESULTS .................................................................................................................... 21
  Experimental Design for Survival .......................................................................... 21
  *myd88*-deficient zebrafish display impaired survival in NF-κB fish lines .......... 22
  Survival experiments to test *myd88*-deficiency in fish lacking fluorescent reporters were inconclusive .......................................................... 23
  Survival experiments to test *myd88*-deficiency in TNF-α fish were inconclusive due to lack of wild type larva ......................................................... 25
  Experimental Design for Imaging ......................................................................... 26
  The presence of a fungal *C. albicans* infection in the hindbrain of
  *myd88* NF-κB fish causes increased activation of the NF-κB pathway ............... 27
  Deficiency of *myd88* does not alter the patterns of NF-κB \ activation in hindbrain infection ................................................................. 29
  Morphogenesis is a virulence factor of *C. albicans* which may account for limited activation of NF-κB or expression of TNF-α ... 31
  Hindbrain images of *myd88* /- TNF-α fish were inconclusive .......................... 32

DISCUSSION ............................................................................................................... 35

LIMITATIONS AND FUTURE DIRECTIONS .......................................................... 43

REFERENCES .......................................................................................................... 46

APPENDIX ................................................................................................................. 52

BIOGRAPHY OF THE AUTHOR ............................................................................... 54
LIST OF FIGURES

Figure i: Reference image for hindbrain injection procedure ........................................... 18

Figure 1: Impaired survival of C. albicans-injected compared to mock-infected myd88 -/- and myd88 +/- NF-κB:EGFP fish .......................................................... 22

Figures 2: Inconclusive survival of C. albicans-injected compared to mock-infected MyD88 +/- and myd88 -/- fish .......................................................... 24

Figure 3: Impaired survival of C. albicans-injected compared to mock-infected myd88 -/- TNF-α:EGFP fish .......................................................... 25

Figure 4: 24 hpi. Low NFκB:EGFP in the hindbrain of mock-infected myd88 +/- fish .......................... 27

Figure 5: 24 hpi. Increased NF-κB:EGFP activation in the hindbrain of C. albicans-infected myd88 +/- fish .......................................................... 28

Figure 6: 24 hpi. Low NFκB:EGFP in the hindbrain of mock-infected myd88 -/- fish .......................... 29

Figure 7: 24 hpi. Increased NFκB:EGFP in the hindbrain of C. albicans-infected myd88 -/- fish .......................................................... 30

Figure 8: 24 hpi. Zoomed-in look at NFκB:EGFP activation in the hindbrain of C. albicans-infected myd88 -/- fish .......................................................... 30

Figure 9: Low NF-κB activation in hyphal C. albicans infection of a myd88 -/- NF-κB fish .......................................................... 32

Figure 10: 24 hpi. Low TNF-α:EGFP in the hindbrain of mock-infected myd88 -/- fish .......................................................... 33

Figure 11: 24 hpi. Inconclusive TNF-α:EGFP activation in the hindbrain of C. albicans-infected myd88 -/- fish .......................................................... 33
LIST OF TABLES

Table 1: Zebrafish lines and references used for this research…………………... 15
Table 2: *Candida* strain and reference used for this research…………………… 16
Table 3: Organization of Confocal Files by Type……………………………………. 52
INTRODUCTION

Overview of fungal infection, specific to *Candida spp. - C. albicans*

It is relatively unknown how many species of fungal microbes exist worldwide at this time. Over 100,000 species have been discovered (52) with predicted numbers up to nearly 5 million yet to be found (19). Commensal organisms of all types help to comprise the human microbiota. A commensal relationship between two organisms describes that in which one organism benefits and the other remains neutral, neither benefits or is harmed. However, there are several examples in which these organisms can actually help the host. With humans, this includes providing digestive enzymes, essential nutrients, and the development of immunity (20). Along the lines of promoting the human immune system, commensal organisms have even been known to influence risk of cancer as well as multiple psychological diseases including major depressive disorder (21). That being said, it is possible for these organisms to also contribute to or cause disease, therefore becoming a pathogen. This switch is largely attributed to changes in a host’s immune function, such as a sudden compromise of immunity. The human immune system is therefore responsible for tolerating commensal organisms, but also to defend against pathogen potential (18). While there could be anywhere up to or beyond 5 million fungal species worldwide, only an extremely small percentage is actually able to become pathogenic in humans, at 0.01% (24). A specific fungal genus, *Candida*, are responsible for being the most pathogenic fungi in a human host. Only 5 species from this group are able to cause disease, including *C. albicans* (25). *Candida albicans* can be found on
anywhere between 40-60% of the population, and resides most commonly in areas such as the gastrointestinal tract, oral cavity, and the vagina. In other words, it is part of the majority of the population’s natural microflora (11). Pathogenicity occurs in this organism’s ability to shift to overgrowth on superficial tissues, which is especially possible in immunocompromised hosts (26). The fungus is able to penetrate the epithelial barrier by morphing from yeast to a more damaging hyphal form, and allows for penetration into deeper tissues, and even the bloodstream (27). *Candida* species such as *C. albicans* are the 4th leading cause of bloodstream infections in the U.S, with mortality rates reaching as high as 50% in extreme cases (28). Disseminated candidemia marks the hyphae penetration into neighboring tissue. In intensive care units (ICUs), *C. albicans* is of utmost clinical significance, and causes as high as 40% mortality in infected patients. *C. albicans* accounts for thousands of deaths annually (6). Although the immune system and fungal commensals have coevolved, dysfunction of human immunity allows opportunistic organisms such as *C. albicans* to become pathogenic. Fungal diseases remain ever prevalent as medical advances aimed to save lives also lead to an increased population of immunocompromised individuals (18). Disseminated infection has been linked to the hospital environment, with invasive procedures providing access to normally sterile compartments within the body (24, 34). Treatments for cancer such as radiation, and a growing HIV/AIDS population also add to the number of people with lowered immunity (5,6). How exactly *C. albicans* is able to evade the defense of the human immune system will be understood by first examining innate immunity.
Overview of the Innate Immune System and *C. albicans* evasion mechanisms

The immune response consists of innate and adaptive immunity. Both systems are interconnected and work together to identify and clear infections. The first step of human immunity is the innate immune system which includes chemical and physical barriers to infection as well as DNA-encoded receptors that recognize common chemical components of pathogens. These are called pattern recognition receptors, or PRRs. More specifically, PRRs are transmembrane proteins encoded in the germ-line. They include ligands which contain an abundance of pathogen associated molecular patterns (41,42).

Anatomical barriers to infection include the epithelial barrier which is composed of the skin and tissues connected to openings of the body. These barriers surround the respiratory, gastrointestinal, and urogenital tracts and help the body to shed pathogens, for example by mucus movement. The innate immune system allows for a quick, non-specific response to infection that then activates the adaptive immune system for a sophisticated clearance. While the latter may take a longer time, it is much more antigen specific. Communication between the two systems is imperative for the cells of innate and adaptive immunity to work together (8).

A pathogen is an organism that causes disease. For humans, these include viruses, eukaryotic parasites, bacteria, and fungi. In order for the body to recognize an invading pathogen, recognition molecules expressed by the host cells must be able to interact with the foreign organisms in some way. Ligands bind to recognition molecules and can be entire pathogens or fragments. These are also known as pattern-associated molecular patterns, or PAMPs, and are recognized by PRRs. The immune system frequently
identifies PAMPs first and uses them to characterize groups of pathogens. Recognition of PAMPs helps continuously recruit immune cells to the site of infection (9). The act of binding ligand to recognition receptor initiates a cascade of events that end in the labeling and destruction of the pathogen, known as the immune response (8).

First responders of the innate immune system are part of the myeloid lineage of cells. This includes all red blood cells, granulocytes, monocytes, and macrophages. Granulocytes fall into four categories: neutrophils, eosinophils, basophils, and mast cells; all of which act quickly in response to infection. Each of them carry many proteins with functions that can directly damage the pathogen, or help with remodeling the tissues where the infection had occurred. Of the four categories, neutrophils constitute the majority of the white blood cells. In response to inflammatory molecules, they swarm to the site of infection in large numbers and work to phagocytize the invader. They can also secrete proteins with a range of antimicrobial effects. Additionally, the myeloid cell lineage gives rise to other phagocytic cells such as macrophages and dendritic cells. These cells have professional antigen-presenting cell (pAPC) function and are key in overlapping into the adaptive immune system by communicating with T lymphocytes after becoming activated by contact with the pathogen. In addition to communication, pAPCs also secrete proteins that activate other immune cells, phagocytose pathogens and present them as peptide antigens on their membrane surfaces. Macrophages are especially specialized phagocytes and efficiently remove pathogens from the site of infection (8).

The method of phagocytosis is important for clearing infection. This is done by cellular uptake of pathogen materials. It is performed mainly by macrophages, neutrophils, and dendritic cells in the tissues. The pathogen is ingested by the cell,
forming the phagosome. Next, the phagosome will fuse with a lysosome which has low pH activated lysosomal enzymes used to kill and digest the pathogen. The digested products are then released from the cell and cleared from the site of infection (8).

The innate immune system, specifically the roles of epithelial barriers and phagocytic cells, comprise the first line of defense against candidiasis, and is a key factor in preventing a disseminated infection (9). *C. albicans* uses a plethora of strategies in order to evade the efforts of the immune system, and even to counter the attempt of clearance (31,32). One of these mechanisms includes epitope masking, where a layer of mannoprotein plays a role in hiding beta-glucan, a cell wall polysaccharide that is a prominent PAMP for detecting *C. albicans* by the Dectin1 receptor. Secreted aspartyl proteinases (SAPs) such as SAPs 9 and 10 degrade complement inhibitors and cleave host antimicrobial peptide histatin (33). Many more mechanisms exist, but perhaps the most important or prominent strategy of *C. albicans* is its morphogenesis abilities. All *Candida* species are pleiomorphic, in that they are able to exist in multiple forms such as yeast, pseudohyphae, and true hyphae. However, *C. albicans* is one of two *Candida* pathogens that is able to be in a true hyphae form (24, 34). Yeast remain unicellular while hyphae become tube-shaped (18). Past experiments have shown that hyphae are better able to invade deep tissue epithelial than yeast cells in reconstructed human tissue models (35,36). The switch to hyphal form triggers additional virulence factors such as upregulation of adhesin genes, specific SAPs, and *Candidalysin* which was recently discovered to damage epithelial cells by forming pores (38, 39). The sheer size and shape of hyphae compared to yeast contribute to the difficulty that phagocytes find in ingesting and killing the fungi (40). Having a basic overview of the innate immune system and
knowledge of the evasion mechanisms exercised by *C. albicans*, the role of specific adaptor protein, MyD88, in fungal infection will be presented.

**MyD88 Dependent Pathways Including NF-κB and IL-1**

PRRs are able to recognize unique fungal PAMPs such as chitin, glucans, and mannans. One family of PRRs, called the Toll-like Receptor group (TLR), were one of the first PRRs to be discovered. These receptors recognize elements of microbes such as peptidoglycan, single and double-stranded RNA, lipopeptides, etc. TLRs have extracellular ligand-binding domains made of leucine-rich repeats (LRRs), and once bound, are induced to dimerize. Adaptors that are TLR-specific include TRIF, TIRAP, TRAM, and MyD88. Following TLR dimerization induced by binding to ligand, signaling is initiated through these adaptors. Signaling pathways of TLRs include the nuclear factor kappa light chain enhancer of B cells (NF-κB) and type I interferons. Activation of a particular pathway depends largely on the protein adaptor(s) that binds to the TLR cytoplasmic Toll/IL-1 receptor (TIR) domain. The NF-κB pathway is important for activating expression of many innate and inflammatory genes (8).

NF-κB is considered a MyD88-dependent signaling pathway (18). After associating with the dimerized TIR domains, MyD88 will initiate a signaling pathway in order to then activate NF-κB. First, MyD88 recruits interleukin 1 receptor associated kinase 1 and 4 (IRAK1 and IRAK4). IRAK1 activates and phosphorylates with tumor necrosis factor receptor associated factor 6 (TRAF6). TRAF6 is then able to create a scaffold that organizes subsequent signaling components. Next, adapter protein
transforming growth factor B activated kinase 1 (TAK1) binding proteins 1 and 2 (TAB1 and TAB2) bring IRAK1 to TAK1 in order to phosphorylate and activate it. Finally, the inhibitor of kB kinase (IKK) complex is recruited which enables TAK1 to phosphorylate and activate IKKB. Subsequently, this last step activates the NF-κB pathway. In the cytoplasm, inactive NF-κB is retained by the inhibitor of NF-κB subunit (IkB), while activated IKK phosphorylates IkB and allows the release of NF-κB into the nucleus. Here NF-κB can activate gene expression and recruitment of innate immune cells to clear infection.

After separating from the IKK complex, TAK1 can also activate the mitogen-activated protein kinase (MAPK) signaling pathway which activates transcription factors Fos and Jun to make up the activator protein 1 (AP-1) dimer. The NF-κB and AP-1 pathways are responsible for activating antimicrobial proteins and peptides, proinflammatory cytokines, and proinflammatory chemokines that are important to the innate immune response.

**Cytokines and TNF-α**

Cytokines are proteins that communicate with cells of the immune system, playing a critical role in cell movement, enzyme activity, cell death, and more. This variety of responses is triggered when a cytokine interacts with its receptor on the target cell. Cell migration may be activated by cytokine-receptor interaction, causing a change in the expression of adhesion molecules or of chemokine receptors that exist on the target cell membrane. An immune cell may also be activated to proliferate, differentiate, or
change effector functions by coming in contact with cytokines, as they are able to signal a cell to increase or decrease a specific enzymes activity level. Instructing cells on when they will survive versus when they will die is another piece of a chemokine’s role.

Cytokines can be put into different families based on their structure and specific roles. One such grouping of cytokines is known as the tumor necrosis factor (TNF) family, and have been shown to regulate cells of the immune system, as well as cells of the skeletal and neuronal systems. TNF-α is a proinflammatory cytokine that is secreted as a soluble protein. It is produced by activated macrophages and lymphocytes as a response to infection, inflammation, and environmental stress and plays a role in stimulating proliferation, differentiation, or activation of different immune cells. TNF-α is an NF-κB-dependent proinflammatory gene, and therefore exists downstream of NF-κB (53). In this way, the activation of NF-κB as well as the expression of TNF-α are dependent on adaptor protein MyD88. Having discussed both important pathways and specific cytokines that interact with MyD88, an introduction to the project at hand will be explained in the context of preliminary data and what is already known on the subject.

**Overview of Project**

By understanding key elements of the innate immune system, it can be determined if the myd88 gene is playing a strong role in response to a foreign invader, especially with fighting candidiasis. The study described here will be largely based on previous work to understand the gene’s function in mice and humans, including experiments in the Wheeler Lab with myd88 mutant fish.
The first aim for this project is to determine if myd88-deficient zebrafish have impaired survival during a systemic hindbrain infection of *C. albicans*, when compared to wild type fish. Several previous studies have answered a similar question by instead looking at mucosal infection as can be demonstrated by a swimbladder infection. The swimbladder shares multiple similarities to the mammalian lung in terms of anatomy and also of gene expression. This allows for the production of a clinically relevant fungal infection model of the human lung (5, 50, 51). Preliminary data collected in the Wheeler lab suggest that myd88-deficient fish have impaired survival when compared to wildtype fish of the same fish line. This would suggest that a localized swimbladder infection proved to be more lethal with the absence of the myd88 gene. The survival of wild type fish was significantly higher than in fish where the gene was knocked out (Archambault and Wheeler, unpublished). Similarly, a study involving wild type and MyD88 mutant mice revealed a difference in survival. The MyD88 mutant mice showed markedly impaired survival in comparison to the wild type group (54). However, counter to these two studies, research conducted on 9 children with mutations in their MYD88 gene did not follow this pattern. Instead, these children showed susceptibility to bacterial infection but remained competent towards fighting off parasitic and fungal infection. This study implies that perhaps the MYD88 gene is only important in fighting bacterial infections rather than fungal in the context of human infection (55). In this project, targeting infections in the hindbrain to exemplify a systemic infection can be compared to preliminary results in the mucosal model.

The second aim of this project was to determine if myd88-deficient zebrafish display decreased NF-κB or TNF-α activation or expression during a systemic hindbrain
infection of *C. albicans*, when compared to wild-type fish. In other words, to determine if NF-κB and TNF-α are *myd88* dependent or independent in fungal systemic infections. Many studies have imaged the activation and expression of different pathways and genes during mucosal zebrafish infections. The first model that was able to image activation of NF-κB and expression of inflammatory gene TNF-α utilized the swimbladder. In high-level infections of *C. albicans*, both localized and tissue-wide epithelial NF-κB activation was visible. Epithelial NF-κB activation was also seen to occur at a significant distance from the infection site. Additionally, in order to measure the downstream effects of increased activation of NF-κB, this study also measured expression of TNF-α, and found that in high-level infections, expression was increased as well (53). Another recently published study also determined significant increased NF-κB activation in *C. albicans*-infected fish versus mock-infected fish. It was also shown that NF-κB activation was more diffuse, where TNF-α expression remained punctuated, keeping close to the actual *C. albicans* cells (18). However, a previous capstone project aimed to specifically determine the importance of the *myd88* gene in recruitment of macrophages to the infection site after a *C. albicans* infection did not provide the expected results. Infections were again administered into the swimbladder of larval zebrafish, causing a mucosal candidemia infection. Under confocal microscope inspection and image analysis, the study did not find any significant differences in macrophage recruitment or fungal burden between wild type and *myd88*-deficient fish (9). This was a curious finding, as it seemed inconsistent with other data suggesting the importance for *myd88* in the defense of fungal pathogens. This data may instead suggest that macrophages are perhaps not as important in fighting fungal infection as other phagocytic cells, like neutrophils. With the
preliminary data in mind, the new direction that this project will focus on is viewing the activation of NF-κB and expression of TNF-α as they relate to myd88 in a systemic infection, rather than mucosal. Hindbrain infections will hopefully be a more accurate representation of disseminated candidemia, and therefore may reveal other roles of myd88.

Whereas swimbladder infections represent a more isolated infection, being more complementary to a mucosal model, hindbrain infections will be utilized in this project with the hopes of determining the difference between the two, and the dangers of a fungal infection once it becomes systemic. Research in the past 10 years has provided the ability to model reliable mucosal, localized, and disseminated infection in zebrafish larvae. This has allowed for countless opportunities in better understanding the parallels between infection models in zebrafish and humans (43,44,45). Previous work with hindbrain infections showed that this route of injection was successful in leading to lethal disseminated candidiasis in larval zebrafish. In severe cases, C. albicans was found throughout the fish in both yeast and hyphal form, even as far away from the initial infection point as the tail (10). The hindbrain ventricle (HBV) is clear, fluid-filled, and normally free of phagocytes. Microinjections are possible as early as 36-48 hours post fertilization (hpf). There is no direct homology between zebrafish and humans for this model, but it still is possible to show a systemic infection, and ease of imaging makes it worthwhile for viewing pathogen-phagocyte interactions as well as different immune pathway activation and expression of a number of genes (18). The best model to provide results for this project are zebrafish larvae, as will be described below.
Zebrafish as a Model

Zebrafish larvae act as a good model organism in research for many reasons. The embryo and larvae are transparent and therefore make injections and imaging much easier to view at any point (3). Fluorescent microscopy is frequently used in order to track specific cells over a period of time. High reproductive rates and quick embryo development allow for a high yield of experiments to take place in a shorter amount of time. Most importantly, in many ways zebrafish are immunologically similar to many mammals, including humans, especially in regards to the innate immune system. At the larval stage, zebrafish rely almost entirely on the innate immune system to handle infections, which allows the unique opportunity to study this system without the overlap of the adaptive immune system. Macrophages and neutrophils are some of the first responder cells in the innate immune system and can be tracked as they move in and out of an infection site. Phagocytosis of the invading pathogen is also viewable in this model, revealing largely how infections are handled and cleared (9). Many established zebrafish lines have been created in order to label cells for tracking. Pathways such as NF-κB as well as inflammatory genes such as TNF-α are visible with EGFP fish lines (46,47). Many models of immune deficiency have also been created within the zebrafish species (48). Finally, the small size and transparency of the host allow for a clinically relevant comparison of disseminated candidiasis between zebrafish and humans (3). The zebrafish is currently the only small vertebrate model being used to study Candida infections. The ability of the transparent zebrafish larvae to allow visualization of infection has deepened
understanding of various immunological questions previously unknown, and has led to new hypotheses to be tested in mammalian models (18).

**Significance of Project**

The fungal microbiome has been largely overlooked in its contribution to the system at large. While the potential for infection from a commensal fungi is relatively low, it is still ever increasing in an age of medical advancement and continued growth of the immunocompromised population. Additionally, fungi are virulent once pathogenic and can cause highly morbid systemic infections. Now more than ever, it is imperative that interactions with fungal commensals are understood. The larval zebrafish model allows for a unique opportunity to visualize the inner workings of the innate immune system, and translate that to mammalian studies. The hindbrain ventricle model is specifically important in testing for antifungal therapies, which could provide enhanced immunity to hosts in the future. Finally, *myd88* is an important signaling molecule for fighting infection. This study hopes to determine the correlation between this importance in mammals and in zebrafish, and seeks to elucidate its function in a systemic hindbrain infection. The role of *myd88* in disseminated candidiasis remains unknown, with this project being a hopeful step in the direction to some discovery.
MATERIALS AND METHODS

Zebrafish Care and Maintenance

The zebrafish used for this experiment are cared for at the University of Maine’s CORE Zebrafish Facility located on campus in Hitcher Hall. Each fish tank is composed of both males and females of their respective line, with continuous water flow at 28 °C. Circadian cycles in the facility followed a 14/10-hour light/dark cycle, and fish were fed with Hikari Micro Pellets (HK40, Pentair Aquatic Ecosystems). Spawns were set up in one of two ways depending on tank availability. The first utilized larger spawning tanks that fit an entire targeted tank. A thin wire netting is set to keep the fish shallow and allow eggs to fall below. The tank is set at a tilt and the fish have the chance to spawn until the following morning. If the larger tanks were already in use, individual spawning tanks were set up with one male and one female per tank. Several smaller tanks were therefore used to set up the fish in the target tank. Those tanks were also set at a slight slant to enhance spawning until the next morning. Fish were generally set up to spawn around 4 pm and then checked up on the following day between 10 am and 4 pm depending on availability. Eggs were collected if available, and the fish were returned to their respective tanks. When the eggs were collected, they were kept at a density of no more than 1 larva/mL either in a 50 or 150mL dish. After collection, the eggs were transferred to fresh E3 media containing 1-phenyl-2-thiourea (PTU, Sigma, 15 mg/mL). Prior to injections, the larvae were kept in a 33 °C incubator and were incubated at 30 °C post injections. The optimal temperature for C. albicans is 37 °C, while the optimal
temperature for zebrafish is between 28-29 °C based on their ability to spawn and develop. Therefore, 30 °C is a comprisable temperature between the two and has been found to have a reliable mortality rate due to infection. This strategy was recommended for this experiment based off of other projects in the Wheeler lab. All use of zebrafish in this project were with accordance to the National Institutes of Health under the Institutional Animal Care and Use Committee (IACUC) protocol A2018-10-01 (2). All training prior to working in the lab was completed and up to date. Zebrafish were treated humanely and were euthanized via Tricaine overdose according to the protocol above.

The zebrafish strains used in this study are listed in Table 2 below.

<table>
<thead>
<tr>
<th>Line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg/myd88hu3568</td>
<td>M. Kanther et al., Gastroenterology, 141: 197–207, 2011, doi: 10.1053/j.gastro.2011.03.042 (57)</td>
</tr>
</tbody>
</table>
Candida Growth Conditions and Preparation

A singular Candida strain was used in this study, listed with reference in Table 1 below (1). Candida was stored at -80 °C in YPD media (DIFCO; 20 g/L peptone, 10 g/L yeast extract) which contains 2% glucose as well as 30% glycerol. Two days prior to injections, the Candida was streaked out onto YPD agar plates for isolated colonies that would grow overnight at 30 °C to keep the temperature constant for the experiments. The following day, a singular colony was mixed into 5 mL YPD liquid and spun in a roller drum wheel (New Brunswick Scientific) overnight, again at 30 °C. On the morning of injections, 500 μL of the overnight Candida culture was isolated and rinsed three times with saline (PBS) solution before being measured by OD600 using the Bio-rad Photometer. Finally, the new solution was then re-suspended for a final density of 5x10^7 cells/mL.

Table 2: Candida strain and reference used for this research

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
</table>

Needle Preparation and Hindbrain Microinjection

In order to prepare the microneedles for injection of the correct concentration, borosilicate capillaries (OD 1.2 mm; ID 0.69 mm) were first pulled into a micro-needle by using a needle puller set to the following guidelines (550 Heat; 0 Pull; 130 Velocity;
110 Time) with adjustments when needed (4). The pulled needles were then filled with 6 μL of PBS using MicroFil fused silicate pipette fillers (World Precision Instruments, Inc, Sarasota, FL) and cut using forceps with the pressure injection system (MPPI etc.) set at the desired pressure. A hemocytometer and immersion oil were used to measure a bolus of PBS at 0.2mm in diameter, and could be adjusted using the pulse duration until it was the correct size. Detailed step-by-step guidelines for microinjections can be found in Gratacap et. al. 2014 (4). Meanwhile, zebrafish were anaesthetized with Tris-buffered tricaine methanesulfonate (160 μg/ml; Tricaine; Western Chemicals, Inc., Ferndale, WA) for 15 minutes before injections. As a control, 15 mutant and 15 wild type myd88 fish were injected with 4 μL of PBS. For the experimental groups, between 30 and 60 mutant and wild type myd88 fish, 48 hpf, were injected with PBS containing Candida-FR (concentration). Injections took place on 2% agarose dishes with between 5 to 10 fish laid on the plate per round. The needles were carefully pushed through the otic vesicle into the hindbrain ventricle of the fish, with the pressure injection system’s pedal being pressed once per injection. Refer to the image below for injection location. Once the injections were completed, those injected with Candida were placed in individual wells of a 96-well plate to be screened for inoculum of 10-25 Candida cells in the hindbrain of each fish. This screening was done using a Zeiss Axiovision VivaTome microscope as described below.
Figure i: Reference image for hindbrain injection procedure, 48 hpf. This image shows a confocal image of a zebrafish’s head. The orange triangle represents the hindbrain ventricle which is the injection destination. The blue circle represents the otic vesicle where the needle punctures through to the inner hindbrain.

Vivatome Screening

2 mL of Tris-buffered Tricaine methane sulfonate (Tricaine; 200 mg/mL; Western Chemicals, Inc., Frendale, WA) was added to 50 mL of E3 media in order to be used to anesthetize the larvae before screening. After 15 minutes, the larvae were taken to the Zeiss Axiobserver Z1 microscope with VivaTome system (Carl Zeiss Microimaging, Thornwood, NJ). Under the scope, the number of individual Candida cells were counted and the location in the zebrafish hindbrain was verified. Individuals with between 10-25 Candida cells correctly injected into the hindbrain were placed into fresh E3 media and kept at 30 °C for the next 3 days. Those that did not meet this criteria were excluded from the remainder of the experiment and euthanized (Tricaine; 800 mg/mL).
Confocal Microscopy

24 hours following the infections, the zebrafish larva of each group, now at 2dpf, were first checked for survival, and then were prepped for confocal imaging. A Tris-buffered Tricaine methanesulfonate (200 μg/mL) solution containing agarose, also known as low melt, was used to position the larva into a 24-well imaging plate. The layout of the imagine plate was four myd88 +/+ PBS injected fish, four myd88 +/- PBS injected fish, eight myd88 +/- C. albicans SC5314 Caf2:FR (1) injected fish, followed by eight myd88 +/- C. albicans SC5314 Caf2:FR (1) injected fish. This layout was done for experiments involving fish of the Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88^{hu3568/hu3568} and myd88^{+/+} lines, and fish of the TgBAC(tnfa:GFP: pd1028Tg) myd88^{hu3568/hu3568} and myd88^{+/+} lines. The fish were randomly selected from the dishes by not observing the individual under the microscope in order to avoid bias when choosing fish. Each fish was placed in the middle of the well with as little liquid as possible, and then the low melt solution was added to fill the innermost circle. With the help of Tricaine in the low melt solution and a small piece of fishing wire, the larva were able to all be gently put into the same position to save time in searching for them at the scope. After they were all lined up, the agarose was left to solidify for about 15 minutes. The microscope used to take images of the fish is an Olympus IX-81 inverted microscope with an FV-1000 laser scanning confocal system (Olympus). The computer software program Fluoview was utilized for capturing these images. With the software, both the dTomato and green fluorescent protein (GFP) fluorescent reporters were selected in order to visualize C. albicans in red and either NF-κB or TNF-α activation in green. They were visualized on a 20x objective with laser filters at 543/610 and 488/510 nm. These filters allowed for excitation and electron
emission. After centering each fish so that the hindbrain was fully visible under the scope, parameters were set on the furthest and closest depths of visibility within the fish’s head. With the minimum and maximum depths set, a series of 2-dimensional image slices were taken spaced 5 nm apart. Anywhere between 20-30 slices were taken per hindbrain image and used to comprise the z stack. For a full fish head image, the stacks were taken with a 20x objective and a 1.1x zoom. For a zoomed hindbrain image, the stacks were taken still with a 20x objective, but a 1.8x zoom to focus on the specific area of interest. Images were saved as .oib files and analyzed using ImageJ. Parameters for the NF-κB channel on ImageJ were set at a minimum of 46 and a maximum of 483, respectively. For the Candida channel, parameters were set at a minimum of 172 and a maximum of 204, respectively. This was kept consistent for all images.

Statistics

Statistical analysis was used to determine significant differences between control and experimental groups in survival curves. Analysis was carried out using GraphPad Prism 7 software (GraphPad Software, Inc., LaJolla, CA). Any significant difference that was recorded from the program is noted in the results below as p-values. Significance was determined by a p-value <0.05, respectively. Kaplan-Meier survival curves were analyzed by the Mantel-Cox test, which is a log-rank. Bonferroni correction was also used to determine if significance existed between the different cohorts of each experiment (Fig. 1, Fig. 2, Fig. 3).
RESULTS

Experimental Design for Survival

A zebrafish line was created for a previous experiment that possessed the mutated *myd88* gene, *myd88*<sup>hu3568</sup>. Having this mutant line as well as wild type *myd88* fish, I was set to start my experiments. For Aim 1, testing survival of the mutant versus wild type fish, I alternated weeks spawning the *myd88*<sup>hu3568</sup> and *myd88*<sup>+/+</sup> lines and the Tg(6xHsa.NFKBN:EGFP) nc1Tg *myd88*<sup>hu3568/hu3568</sup> and *myd88*<sup>+/+</sup> lines. This is because zebrafish should not be spawned back to back weeks. Two weeks between experiments is adequate time for the fish to recover before spawning again. In a typical experiment, genotyped *myd88*<sup>+/+</sup> wild type or *myd88*<sup>-/-</sup> fish were in-crossed, and the resulting eggs were collected the following morning. Around the 24 hours post collection, at the prim25 stage, the larvae were dechorionated and injected with a prepared concentration of 5.0x10<sup>7</sup> cfu/mL fluorescent *Candida* (CAF2-dTomato) in 5% PBS. 4 nL of the inoculum was injected into each larva’s hindbrain. Around 30-50 fish of each group, mutant and wild type, were injected with *C. albicans* SC5314 Caf2:FR. For both groups, about 15 fish were also injected with 4nL of PBS as a control. After injections, each larva was screened for the correct number of inoculum (10-25 *Candida* cells) and correct location in the hindbrain. The survival of the fish was then followed over the next three days, with the death of a fish determined by no visual heartbeat. Survival data was plotted as Kaplan-Meier and analyzed for statistics on Graphpad.
myd88-deficient zebrafish display impaired survival in NF-κB fish lines

Following the injections and screening described in the methods of this project, the larva were kept with their respective groups for the next 72 hours in order to track their survival. There were five experiments involving fish of the Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88^{hu3568/hu3568} and myd88^{+/+} line, in which NF-κB activity drives expression of enhanced green fluorescent protein (EGFP). The four groups per experiment were as follows: ~15 PBS injected myd88^{+/-} NF-κB, ~15 PBS injected myd88^{-/-} NF-κB, ~30 *C. albicans* injected myd88^{+/-} NF-κB, and ~30 *C. albicans* injected myd88^{-/-} NF-κB. The ability to attain all four groups and the desired number of fish for each group depended on the spawns. In some cases, the number of fish had to be lowered for each group, or some groups were left out entirely. All of the data from each experiment was collected and pooled into a representative survival plot, shown here.

**Figure 1: Impaired survival of *C. albicans*-injected compared to mock-infected myd88^{-/-} and myd88^{+/-} NF-κB:EGFP fish.** Pooled data from all myd88 NF-κB experiments. This figure represents a total of 5 experiments, and 300 fish. This involved 45 PBS injected myd88^{+/-} NF-κB fish, 44 PBS injected myd88^{-/-} NF-κB fish, 73 CAF2 FR injected myd88^{+/-} NF-κB fish, and 138 CAF2 FR injected myd88^{-/-} NF-κB fish. Overall, myd88-deficient NF-κB fish showed significantly impaired survival when compared to the wild type (p < 0.0001).
From these results, we can conclude that for a systemic fungal infection of *C. albicans*, NF-κB fish that lacked adaptor protein *myd88* displayed significantly impaired survival when compared to NF-κB fish that contained *myd88*. This would suggest that the *myd88* gene is an important factor in protecting fish from systemic fungal infection. This will be further explored in the Discussion section.

Survival experiments to test *myd88*-deficiency in fish lacking fluorescent reporters were inconclusive.

As fish lines were spawned bi-weekly for zebrafish health, non-transgenic *myd88* and TNF-α *myd88* lines were also used to track survival. Two experiments involving non-transgenic *myd88* fish, lines *myd88hu3568/hu3568* and *myd88*+/+, took place and involved four groups: ~15 PBS injected *myd88*+/+, ~15 PBS injected *myd88*−/−, ~30 *C. albicans* injected *myd88*+/+, and ~30 *C. albicans* injected *myd88*−/−. Based on a weak spawn in the first experiment, it was not possible to infect a CAF2 FR injected *myd88*−/− group. The two experiments therefore varied in results, and are shown here.
Figures 2: Inconclusive survival of \textit{C. albicans}-injected compared to mock-infected \textit{myd88} +/- and \textit{myd88} -/- fish. Experiments involving the non-transgenic \textit{myd88} fish line. Experiment one on the top involved 46 fish: 15 PBS injected \textit{myd88} +/-, 15 PBS injected \textit{myd88} -/-, 16 CAF2 FR injected \textit{myd88} +/-, and no CAF2 FR injected \textit{myd88} -/- . Experiment two on the bottom involved 109 total fish: 17 PBS injected \textit{myd88} +/-, 17 PBS injected \textit{myd88} -/-, 34 CAF2 FR injected \textit{myd88} +/-, and 41 CAF2 FR injected \textit{myd88} -/- . Based off of these two experiments it is unclear if the \textit{myd88} gene played a role in survival. No significance was found for either experiment between wild type and \textit{myd88}-deficient fish. (Pooled from both experiments : p = 0.9836, Experiment 11/13/19 only: p = 0.6351)

These results show that there was no difference in the survival between the wild type and mutant fish, but potential technical issues are apparent due to the high mortality in PBS injected/mock infected fish.
Survival experiments to test myd88-deficiency in TNF-α fish were inconclusive due to lack of wild type larva.

Two experiments with myd88 TNF-α fish were also performed. While the typical four experimental groups were desired as with the plain and NF-κB lines, wild type TgBAC(tnfa:GFP: pd1028Tg) myd88+/+ fish never spawned. Therefore, the two groups for each of these experiments were: ~15 PBS injected TgBAC(tnfa:GFP: pd1028Tg) myd88hu3568/hu3568 fish, and ~30 C. albicans SC5314 Caf2:FR injected TgBAC(tnfa:GFP: pd1028Tg) myd88hu3568/hu3568 fish. The results of these two experiments were pooled and are shown in the survival curve below.

Figure 3: Impaired survival of C. albicans-injected compared to mock-infected myd88 -/- TNF-α:EGFP fish. Combined experiments for myd88 -/- TNF-α fish. The first experiment, on 2/27/20, involved 63 fish: 13 PBS injected myd88 -/- TNF-α fish, and 50 CAF2 FR injected myd88 -/- TNF-α fish. The second experiment, on 3/12/20, involved 31 fish: 10 PBS injected myd88 -/- TNF-α fish, and 21 CAF2 FR injected myd88 -/- TNF-α fish. From the figure, it is apparent that fish injected with PBS had a much higher survival rate than fish injected with C. albicans. There was a significant difference between the mock-infected and C. albicans-infected fish (p < 0.0001).

Given the data above, conclusions can be drawn about the difference in survival of the PBS injected and CAF2 FR injected fish of the same line. With PBS injected fish
showing significantly higher survival than *C. albicans* injected fish, this pattern follows results collected from the NF-κB survival experiments. However, without a successful spawn from *myd88* +/- TNF-α fish, there is no wild type group to compare the mutant data to. Without that comparative data, no conclusions can be made about the *myd88* gene’s role in survival for *myd88* TNF-α fish.

**Experimental Design for Imaging**

In order to address the second aim of this thesis, determining if *myd88*-deficient zebrafish display decreased NF-κB or TNF-α activation during a systemic hindbrain infection of *C. albicans*, when compared to wild type fish, confocal microscopy was used to image the zebrafish hindbrain 24 hours post infection (hpi). The goal of imaging these infected fish by confocal microscopy was to determine the relationship between the *myd88* gene and the activation of innate immune pathways such as NF-κB and cytokine family TNF. This was possible due to the fish lines Tg(6xHsa.NFKBN:EGFP) nc1Tg and TgBAC(tnfa:GFP: pd1028Tg) driving expression of the EGFP fluorescent protein either when the NF-κB signaling pathway is active or when the TNF-α gene is being expressed under the correct settings on the microscope. Images were taken following the Confocal Microscopy methods stated above, and images were obtained of all groups of the NF-κB fish and mutant groups only for the TNF-α fish. With the help of ImageJ, each picture was analyzed and the following conclusions were made possible.
The presence of a fungal *C. albicans* infection in the hindbrain of *myd88* NF-κB fish causes increased activation of the NF-κB pathway.

Images were taken for the same four groups of Tg(6xHsa.NFKBN:EGFP) nc1Tg *myd88*hu3568/hu3568 and *myd88*+/+ fish as were standard for the survival curve data. Due to the time it takes to image each individual fish, four fish for each PBS group, and eight fish for each CAF2 FR group were imaged at a time. Specifically focusing on Tg(6xHsa.NFKBN:EGFP) nc1Tg *myd88*+/+ fish, a total of 4 PBS injected and 8 *C. albicans* injected fish were imaged. A representative picture of each group is shown below.

**Figure 4: 24 hpi. Low NFκB:EGFP in the hindbrain of mock-infected myd88 +/- fish.** Outlined in the dotted orange triangle is the location of the hindbrain ventricle. The arrow will continue to mark the location of the hindbrain throughout the following figures. Confocal microscope image of a PBS injected *myd88* +/- NF-κB fish 24 hpi. Going from left to right, the first image shows all channels turned on, so that the fish itself, the NF-κB pathway, and any *C. albicans* would be lit up. The second image tracks channels 1 and 2 only, channel 1 being NF-κB, and channel 2 being *C. albicans*. The third image tracts channel 2 only. Being a PBS injected fish, there would be no *C. albicans* viewed in these images. Activation of the NF-κB pathway is not present in the hindbrain for PBS injected fish.
Figure 5: 24 hpi. Increased NF-κB:EGFP activation in the hindbrain of *C. albicans*-infected *myd88* +/- fish. This image represents two CAF2 FR injected *myd88* +/- NF-κB fish. The ordering of channels are the same as in Figure 4. Here there is clear activation of NF-κB in the hindbrain, especially surrounding the infection site, but also occasionally in the epithelium or even further away.

To assess the level of activation a hindbrain fungal *C. albicans* infection triggers, the two groups above can be compared. With the PBS injected fish, there was no noticeable activation of NF-κB in the hindbrain, seen by the clear lack of green lighting up that area. However, when the infections were done with *C. albicans* instead, there is noticeable upregulation of the NF-κB pathway now in the hindbrain. In the hindbrains of infected fish, there are bright green cells surrounding the fungi shown in red. It is worth noting as well that 2 out of 4 fish in this group showed patterns of NF-κB similar to Figure 5, Row 1, while 2 out of 4 fish in this group showed patterns of NF-κB similar to Figure 6, Row 2. The pattern of Figure 5, Row 1 shows a localized NF-κB response, remaining around the infection site, where the pattern of Figure 5, Row 2 shows a more diffuse NF-κB response, where activation is seen in the epithelial cells beyond the infection site. From these images it can be concluded that the presence of a fungal
infection in the hindbrain of myd88 +/- NF-κB zebrafish prompts the immune system to turn on the NF-κB pathway, with different patterns in different individual fish.

Deficiency of myd88 does not alter the patterns of NF-κB activation in hindbrain infection

Images were also taken of PBS and C. albicans injected Tg(6xHsa.NFKBN:EGFP) nc1 Tg myd88<sup>hu3568/hu3568</sup> groups in order to determine the role of myd88. As with the wild type fish, four mutant PBS injected and eight mutant CAF2 FR injected fish were imaged and analyzed using ImageJ. There were a total of 8 PBS injected myd88 +/- NF-κB fish and a total of 16 CAF2 FR injected myd88 +/- NF-κB fish. Due to limitations from the availability of the confocal microscope and the time required for imaging each fish, only a small portion of injected fish were imaged. In addition to images taken at a zoom of 1.1, a few images of the CAF2 FR injected mutant fish were also imaged at a zoom of 1.8 in order to view the hindbrain more clearly. Representative images for the mutant group are shown below.

![Representative images for the mutant group](image_url)

**Figure 6: 24 hpi. Low NFκB:EGFP in the hindbrain of mock-infected myd88 +/- fish.** This image represents a PBS injected myd88 +/- NF-κB fish. The ordering of channels are the same as in Figure 4. Here there is no activation of NF-κB in the hindbrain, similar to the results seen in the myd88 +/- NF-κB fish images.
Figure 7: 24 hpi. Increased NFκB:EGFP in the hindbrain of C. albicans-infected myd88 −/− fish. This image represents two fish from the group CAF2 FR injected myd88 −/− NF-κB fish. Both fish have a zoom set at 1.1. In these fish there is clear NF-κB activation surrounding the infection and in some cases, in the epithelium and further away (bottom row).

Figure 8: 24 hpi. Zoomed-in look at NFκB:EGFP activation in the hindbrain of C. albicans-infected myd88 −/− fish. This image represents the same two fish from Figure 7 but with a zoom of 1.8 for each to show more clearly the hindbrain. Here it is easier to see phagocytic cells.

From the images above, several conclusions can be made. First, there is clear activation of the NF-κB pathway when the fungal infection is present. As with the wild type groups, fish that were myd88-deficient also had a lack of NF-κB activation within
the hindbrain in PBS injected fish, but increased activation with the *C. albicans* injected fish. From this similarity of pattern, it seems that activation of the NF-κB pathway in the hindbrains of larval zebrafish fungal infections is *myd88*-independent. This means that the regulation and activation of the pathway acted independently of whether or not the *myd88* gene was present. A final conclusion that can also be made was that from looking at Figure 7 and Figure 8, it is seen again the difference in the location of NF-κB activation. In Figure 7, NF-κB is more localized around the actual *C. albicans* cells, whereas in Figure 8, there is also strong activation in the epithelium as well as further from the infection site. Since this was also recorded in *myd88*+/+ NF-κB fish, the variability in NF-κB activation was also independent of *myd88*. These results are based on the images of less than 15 fish per group, and therefore should be further investigated for accuracy in additional fish.

**Morphogenesis is a virulence factor of *C. albicans* which may account for limited activation of NF-κB or expression of TNF-α**

In one infection involving a Tg(6xHsa.NFKBN:EGFP) nc1Tg *myd88*hu3568/hu3568 fish, there was notable hyphal growth out of the hindbrain and into the forebrain. As was previously stated, the ability of *C. albicans* to switch from yeast to hyphae form is an important mechanism for evading the host immune system. Hyphae are able to destroy phagocytes and breach epithelial barriers to spread to further tissues. The purpose of the image below is to spark discussion as to why activation of NF-κB seems to decrease around the hyphae cells, and only increase in the epithelium. This image will also be
compared with the resulting TNF-α images, and expanded upon in the Discussion section.

**Figure 9: Low NF-κB activation in hyphal *C. albicans* infection of a *myd88* -/- NF-κB fish.** This infection generally moved almost completely into the forebrain before bursting through the epithelium of the fish. There are lots of NF-κB activation throughout the epithelial cells, but hardly any surrounding the actual infection anymore. Here the hindbrain ventricle is outlined in orange again to emphasize the location of the infection moving into the forebrain.

Hindbrain images of *myd88* -/- TNF-α fish were inconclusive

Finally, images were also taken of the TgBAC(tnfa:GFP:pd1028Tg) *myd88*hu3568/hu3568 fish used in experiments. As with the survival curves, data collection was limited by the lack of success of the wild type TNF-α fish to spawn. In order to hopefully at least determine the difference in activation between PBS injected *myd88* -/- TNF-α fish and CAF2 FR injected *myd88* -/- TNF-α fish, 4 PBS fish were imaged and 2 CAF2 FR fish were imaged as well. Examples of these two groups are shown below.
Figure 10: 24 hpi. Low TNF-α:EGFP in the hindbrain of mock-infected *myd88* -/- fish. This image represents a PBS injected *myd88* -/- TNF-α fish. The ordering of channels are the same as in Figure 4. Here there is noticeable but little activation of TNF-α in the hindbrain area, and no activation anywhere else in fish’s head.

Figure 11: 24 hpi. Inconclusive TNF-α:EGFP activation in the hindbrain of *C. albicans*-infected *myd88* -/- fish. Row 1 and 2 represent two different fish of the same group: CAF2 FR injected *myd88* -/- TNF-α fish. The ordering of channels are the same as in Figure 4. There is some activation of TNF-α for both fish in the epithelium, but no noticeable activation within the hindbrain or near the infection site at all.

The comparison of *myd88*-deficient fish of the PBS and CAF2 FR group is less clear than it was for the NF-κB fish. It is clear that not only do the two group’s images look significantly different from each other, but also that the CAF2 FR injected group’s images look significantly different from any other images taken on the confocal microscope for this project. Reasons why this may be will be elaborated extensively in the discussion, but essentially a block of time passed between when the PBS fish were imaged and when the CAF2 FR fish were imaged for the *myd88* -/- TNF-α fish, which
may account for the differences in appearance between the two groups. For this reason, the two groups cannot be compared in confidence. The lack of wild type groups to look at too further limits the conclusions that can be drawn about *myd88*’s role in activating TNF-α in the hindbrain during a hindbrain fungal infection.
DISCUSSION

In this thesis project, we investigated the role of the well-conserved *myd88* signaling protein in the zebrafish innate immune response to *Candida albicans*. In this first study, we also found that *myd88* plays an important role in protecting against hindbrain infection, similar to previous results from our lab suggesting that it is important in protection against mucosal infection (Archambault and Wheeler, unpublished). Furthermore, we were intrigued to find that the NF-κB pathway is activated in hindbrain infection, and that its activation appears independent of functional *myd88*. This suggests that *myd88* plays a selective role in immunity to *C. albicans* in this model, which is consistent with its known role in protection against fungal infection in mice but a lack of association between inactivating mutations and fungal infection in humans. This discussion will focus largely on how the results of this project compare to past research done in the lab and what is already known about the role of *myd88* in systemic fungal infections. Any possible reasoning for the data will also be included as well as suggestions for future investigation. The flow of this discussion will follow the ordering of the results, with additional points for consideration added at the end.

The first aim of this project was to determine the role that *myd88* played in the overall survival of zebrafish during a systemic fungal *C. albicans* infection. This was done using several fish lines including plain *myd88* +/+, and +/- fish, *myd88* +/+ and +/- NF-κB fish, and only *myd88* -/- TNF-α fish. Each experiment strived to include four groups, two involving injections of PBS into both wild type and mutant fish, and two involving injections of the *C. albicans* strain CAF2 FR into both wild type and mutant
fish as well. Survival curve results utilizing the myd88 NF-κB line showed conclusive and promising results. A total of five experiments took place with adequate total fish numbers for each group. By pooling the data from each experiment, a single representative curve was created (Fig. 1). Analysis on Prism yielded a significant difference between survival of myd88 +/+ NF-κB fish injected with C. albicans and myd88 -/- NF-κB fish injected with C. albicans. This suggests that the presence of the myd88 gene plays an important role in systemic fungal infections. Being an adaptor protein responsible for recruiting immune cells to the site of infection, it makes sense that the myd88-deficient fish showed decreased survival in its absence. This result supports the initial hypothesis that myd88 aids in innate immunity against fungal pathogens. It is also consistent with the preliminary findings in the Wheeler Lab, showing that in the swimbladder mucosal model, survival of myd88-deficient fish were significantly impaired when compared to the wild type (Archambault and Wheeler, unpublished). It would be interesting to compare the percent survivals between these two studies to determine if there was a difference between the swimbladder and hindbrain models.

Survival curve results of the remaining fish groups, however, were not as clear. Non-transgenic myd88 +/- and -/- fish were run on alternating weeks with NF-κB, and utilized the same procedure. Only two experiments yielded interpretable results (Fig. 2). The two survival curves are immediately concerning, as the survival of PBS fish, whether lacking myd88 or not, are seen to decrease over the 72 hours. Being a neutral saline solution, PBS is used for control groups as it does not itself cause mortality when injected correctly into zebrafish. Close to 100% survival of PBS groups can be evidence of both good basal health of the fish larva as well as reliability of experimental injections. It is
therefore possible that injection error occurred. However, in this case, for both plain
myd88 fish experiments, notes were made about the larval appearance during
dechorionation. The embryos did not act responsive to the process of being released from
their protective eggs, and did not display much movement. Furthermore, many of them
did not survive the process of injections (being sedated and maintaining health on the
injection dish). Had this been only one of the experiments it could have been related to
adding too much Tricaine to the pre-injection dish, or taking too long with injections so
that the fish dried up on the dish. While this is still possible, the fact that this was noted
for both non-transgenic fish experiments and not for any of the NF-κB experiments, it is
unlikely that this is what caused the low survival during injections. It would have been
helpful to have taken images of the non-transgenic larva to compare them to the NF-κB
and TNF-α larva pre-injections for this purpose. Whatever the exact reason may be, with
lowered survival of both wild type and mutant PBS injected groups, the survival of the
CAF2 FR injected groups should not be relied upon. While it would have made sense to
see the same pattern as with the NF-κB fish, where the lack of myd88 resulted in lowered
survival, the conclusion cannot be made for this fish line.

The last experiments to test for myd88’s role in survival of systemic fungal
infections incorporated fish from the line myd88 TNF-α. Unfortunately, only mutant
myd88 -/- TNF-α fish spawned over the duration of this project. Reliability in that PBS
injected mutant fish had close to 100% survival over all can be used to suggest that the
percent survival of the CAF2 FR injected groups was accurate. Both experiments also
yielded similar percent survival of the infected fish after 72 hours, further prompting the
accuracy of that data. However, without having the wild type groups to compare with, no
conclusions can be made about the role of myd88 in the survival of TNF-α fish. It can be hypothesized that they would show the same pattern as NF-κB fish given that the fact that containing GFP should not affect the survival of the fish, and thus would not change the results.

For both plain and TNF-α fish lines, it would be beneficial to continue experiments that test their survival with or without myd88. The plain fish used should possibly be spawned and inspected for health, and potentially future experiments could utilize different tanks of that line. TNF-α and NF-κB fish were spawned and saved to grow up during the process of this project as well, and hopefully can be utilized in the future to confirm the results found now. Perhaps myd88 +/+ TNF-α fish growing up now will be useful to spawn in the next year and additional experiments can be made.

The second aim of this project was to determine the role that myd88 plays in the activation of NF-κB and TNF-α during a systemic fungal infection. This was determined by confocal microscopy images taken 24 hpi for fish of these specific lines. Again, each imaging period strived to include four groups, two involving injections of PBS into both wild type and mutant fish, and two involving injections of the C. albicans SC5314 Caf2:FR into both wild type and mutant fish as well. For the TNF-α fish, this was not possible due to the lack in spawn from wild type myd88 fish. Additionally, experimental time was cut short due to the COVID-19 pandemic.

Activity in the hindbrain of NF-κB yielded especially interesting and conclusive results. It was initially hypothesized that myd88-deficient fish would show significantly less activation of NF-κB since myd88 has been proven to be essential in activating signaling pathways that recruit immune cells to the infection site (53). Without this
adaptor protein, *myd88*-deficient fish would be expected to show reduced activation. However, previous studies with the recruitment of macrophages showed that there was no difference between fish of the wild-type or mutant groups (9). When studying the images of the *myd88* +/+ NF-κB groups from both PBS and CAF2 FR injected fish, there is noticeable increased activation with the presence of a *C. albicans* infection. The activation of NF-κB in the zebrafish hindbrain during a systemic fungal infection is not something that has been looked at before. These results suggest that while there is no basal level of NF-κB within the hindbrain, upon injection of a fungal pathogen, there is significant upregulation. *Candida* species are known to activate both NF-κB and TNF-α signaling components (53). With previous work done in the Wheeler lab it had been determined that both NF-κB and TNF-α were expressed in mucosal *C. albicans* infection modeled in the zebrafish swimbladder. At basal levels, NF-κB activation was seen in several tissues, but not the swimbladder. It was not until an infection took place that NF-κB was seen within the swimbladder, in the epithelium nearby, and even further from the site (18). These results are agreeable with the images taken for this project as well.

Therefore, in both systemic and mucosal models, a fungal infection by *C. albicans* triggers the activation of NF-κB. With that in mind, there was still a small number of individual fish for each group, and future experiments should take place to confirm these results.

Keeping with the NF-κB images, the next conclusion to consider is the comparison between activation seen in wild type versus mutant *myd88* fish. As stated before, it was expected that being *myd88*-deficient would impair the activation of NF-κB in some way. However, a recent capstone project conducted in the Wheeler Lab that
aimed to conclude similar results with macrophage recruitment of mucosal infection did
not find it to be the case. During that research, swimbladder infections took place in a fish
line that had fluorescent macrophages. Recruitment was determined in the four
experimental groups that were used for this current project as well. Using the Confocal
Microscope, it was determined that there was no significant difference in the recruitment
of macrophages for fish that were myd88-deficient and fish that did contain the gene.
Focusing back to this project, there is a large difference due to the infection now being
within the hindbrain, and representing a systemic infection rather than mucosal. With this
in mind, it was initially hypothesized that NF-κB activation should be different between
the two experimental groups. Comparing Figures 4-8 though, it is seen that this again was
not the case. There was no apparent difference between the activation level of NF-κB in
myd88 +/+ or -/- fish. The same patterns were seen in both PBS groups, being no basal
activation, and in CAF2 FR groups, being increased activation. While the initial
hypothesis implied that there may be a difference due to the severity of the infection
macrophages are one of the major cell types recruited by the NF-κB pathway, so if it was
previously found that their recruitment levels did not decrease without myd88, it is not
too surprising that the NF-κB pathway activity did not either. This leads to the conclusion
that during a systemic C. albicans infection, NF-κB activation acts independently of
myd88. It is activated regardless in order to fight the infection. Additional experiments
are needed to support or refute our hypothesis.

When interpreting the confocal images from the TNF-α:EGFP fish, few
conclusions could be made. This was in part due to the lack of spawning with the myd88
+/+ TNF-α fish, and in part due to the differences in the images that were taken between
PBS and *C. albicans* injected myd88 -/- TNF-α fish. With this first obstacle, there is not much to discuss. While spawning was attempted for the wild type line, myd88 +/- TNF-α were also being spawned and genotypes for future use of the lab. Hopefully this will provide less trouble spawning in coming experiments, as there will be more fish to utilize for this purpose. With the second obstacle, multiple factors may have played a role in the different outcomes of the photos. The images of the mock-injected fish were taken at the same time the images of the different NF-κB groups were being taken. However, with multiple labs sharing the microscope, substantial time passed before the *C. albicans*-injected fish were imaged due to another researcher’s time overlapping. This change in projects the microscope was used for may have changed the settings between when the first and last images were taken. Another possibility, perhaps more likely, is that the fish dried up on the well plate as time passed, and therefore turned out looking differently. With all of that being said, it would be interesting to continue these survival experiments and confocal imaging for TNF-α fish. It had been shown in previous research in the Wheeler Lab that TNF-α was in fact activated in the presence of *C. albicans* in a swimbladder infection (53). TNF-α was seen to be upregulated especially around the infection site and showed a clear increase from mock-infected fish (18). By testing this in terms of a hindbrain infection and also with the presence and absence of *myd88* it could show both how TNF-α responds to a potential systemic fungal infection, and also if it is *myd88*-dependent or independent in this context.

While this was not necessarily a goal of this project nor purposely tested for, another potential future question to expand on is that of the activation of NF-κB and TNF-α in terms of *Candida* form. It has been seen previously that NF-κB activation
actually decreases with increasing *Candida* hyphal growth versus yeast cells (38-40). As is known by the mechanisms of *C. albicans*, this form switching is an important virulence factor for the fungi to escape phagocytosis and cause further damage to the host. Therefore, it would make sense that an increase in hyphae would correspond with a decrease in successful phagocytosis or even phagocytes in general, as they are lysed by the hyphal growth. When looking through the images from this project, there was one fish of the NF-$\kappa$B group with a notable hyphal explosion, as seen in Figure 9. It is interesting to view this image in comparison to Figures 5, 7, and 8, all showing infected NF-$\kappa$B fish. For those previously discussed, there is a clear increase of NF-$\kappa$B activation between the mock-infected and the *C. albicans* infected groups. However, in this case where there are filamentous *C. albicans* cells from hyphal growth throughout the fish’s hindbrain and forebrain, there is no longer NF-$\kappa$B activation around the infection site hardly at all. While extremely vibrant in the epithelial cells where the *Candida* is breaching the constraints of the host’s body, it seems like where phagocytic cells were once lit up green as well, they have given up the fight or have been destroyed. This can also be compared in the context of Figure 10. Perhaps the resulting images of the TNF-$\alpha$ fish were not in fact affected by the time it took to take them, but were instead affected by the clear amount of hyphae in each fish. Unfortunately, the two examples in Figure 10 were the only images taken from the experimental group, and cannot be representative as a whole. Both fish had clear explosive infections involving majority hyphae versus yeast. It could be that the limited activation of TNF-$\alpha$ that is seen in those images is due to the *C. albicans* form switching mechanism. For both NF-$\kappa$B and TNF-$\alpha$ fish, this would be interesting to examine further and determine for sure.
LIMITATIONS AND FUTURE DIRECTIONS

There were limitations that may have affected the results of this thesis. A majority of the methods for this project were based off of past research done in the Wheeler Lab, specifically by Linda Archambault and Drew Brooks. Both researchers worked with myd88 fish lines as well as swimbladder infections with *C. albicans*. The purpose of this project was largely to replicate these preliminary findings in terms of a systemic infection demonstrated by hindbrain injections. It has been previously studied that early response of the immune system is highly correlated with the survival of the fish. Therefore, it is beneficial to image fish between 4-6 hpi, in order to view the primary stages of innate immunity (10). Due to the extensive time that the injection process takes, I was not able to return to the lab 4-6 hours later in order to image the fish. My schedule allowed me to set up spawns on Wednesday’s and streak *Candida*, create overnight cultures on Thursday’s and collect embryos, and then spend the majority of the day on Friday’s going through the whole injection process. Usually on Friday’s I would be in the lab injecting and screening fish until 5 pm due to breaks being taken to attend other classes. With that being the case, I would have had to come back to the lab between 9 and 11 pm in order to image the fish at their prime time. This was not possible for me this semester, and so the fish for this project were imaged 24 hpi instead of 8 hpi. Drew Brooks also imaged his fish 24 hpi which seemed to give reasonable results, as he did not mention difficulty with imaging at that time. The limited time also limited the amount of fish I was able to image on the confocal microscope. While some results appear hopeful,
mostly in regards to the NF-κB fish, they should still be replicated in the future to have larger numbers representing each group.

Perhaps the greatest limitation to this experiment was recognizing the difference between a systemic versus a localized infection. By administering the injections into the hindbrain of the zebrafish larvae, I was hoping to demonstrate a systemic infection model instead of the mucosal infection model that had been done before using swimbladder infections. Throughout the project, I did not really consider whether or not my injections would cause a systemic infection, I more so assumed they were just by being in the hindbrain. However, after viewing the confocal images with my Advisor, Dr. Robert Wheeler, he brought up the fact that these infections look to be localized. A systemic infection becomes highly lethal because it is able to get into the bloodstream, and therefore travel throughout the whole body of the host. If this were the case for my infections, there may have been \textit{C. albicans} cells throughout the zebrafish, rather than localized in the hindbrain, the point of infection. If that were the case, then I would have been able to more properly address my thesis in terms of a systemic infection, rather than a localized hindbrain infection. That being said, it may be more appropriate to view my project in terms of a localized hindbrain fungal infection rather than systemic. Since hindbrain injections have been shown to cause systemic infection, it may have been that the fish were too old at the time of my injections, and therefore the barrier between the hindbrain and the bloodstream had closed off, where it usually remains open at the prim 5 stage.

There are many directions that future researchers may take this preliminary data. Activation of pathways such as NF-κB and expression of genes such as TNF-α have not
been studied extensively in the hindbrain of zebrafish. Knowing their basal and post-infection activation levels gives insight into how important they become and how well they are recruited in the context of any type of pathogen that may be used. Where I decided to focus on the MyD88 adaptor protein, there are numerous players in the innate immune system that may have major or minor roles with infectious diseases. This study should be replicated in order to confirm the survival patterns of myd88-deficient fish, and also to understand the relationship between myd88 and the expression of TNF-α in mock-infected and C. albicans-infected fish. Furthermore, since this experiment did not appropriately address the definition of a systemic infection, but rather a localized brain infection, it would be interesting to see how the results in this paper would compare to future systemic models. Whether my results related more closely to mucosal infection in the swimbladder, or if they did portray a systemic infection despite the lack of spread seen. Future projects could compare imaging fish at 4-6 hpi as well as 24 hpi to determine how important that is to the activation and expression of NF-κB and TNF-α. Furthermore, to build off of all of this, it would certainly be interesting to determine what specific cell types are being recruited to either a localized hindbrain infection or a systemic infection (macrophages, neutrophils, etc.) Overall, by expanding or branching off of this project, more mysteries can be solved about the immune system of zebrafish which can be transitive to humans and provide insight on better mechanisms for preventing and treating numerous infections.
REFERENCES


**Table 3: Organization of Confocal Files by Type** – for use of the Wheeler Lab

<table>
<thead>
<tr>
<th>File Name</th>
<th>Date</th>
<th>Genotype</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2120A1</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sup&gt;++&lt;/sup&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>2120A4</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sup&gt;++&lt;/sup&gt;</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>2120A5</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>2120B1</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sup&gt;++&lt;/sup&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>2120B2</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>2120B4</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sup&gt;++&lt;/sup&gt;</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>2120B5</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>2120C4</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sup&gt;++&lt;/sup&gt;</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>2120C5</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>2120C6</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>2120D4</td>
<td>2/1/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sup&gt;++&lt;/sup&gt;</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>31420A1</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>31420A2</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>31420A3</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>31420A4</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>31420B1</td>
<td>3/14/20</td>
<td>TgBAC(tnfa:GFP: pd1028Tg) myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>31420B2</td>
<td>3/14/20</td>
<td>TgBAC(tnfa:GFP: pd1028Tg) myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>31420B3</td>
<td>3/14/20</td>
<td>TgBAC(tnfa:GFP: pd1028Tg) myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
<tr>
<td>31420B4</td>
<td>3/14/20</td>
<td>TgBAC(tnfa:GFP: pd1028Tg) myd88&lt;sub&gt;hu3568/hu3568&lt;/sub&gt;</td>
<td>PBS</td>
</tr>
</tbody>
</table>
Table 3 (continued)

<table>
<thead>
<tr>
<th>File Name</th>
<th>Date</th>
<th>Genotype</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>31420C1</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88^hu3568/hu3568</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>31420C2</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88^hu3568/hu3568</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>31420C3</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88^hu3568/hu3568</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>31420C4</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88^hu3568/hu3568</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>31420D1</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88^hu3568/hu3568</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>31420D2</td>
<td>3/14/20</td>
<td>Tg(6xHsa.NFKBN:EGFP) nc1Tg myd88^hu3568/hu3568</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>31420E1</td>
<td>3/14/20</td>
<td>TgBAC(tnfa:GFP:pd1028Tg) myd88^hu3568/hu3568</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
<tr>
<td>31420E4</td>
<td>3/14/20</td>
<td>TgBAC(tnfa:GFP:pd1028Tg) myd88^hu3568/hu3568</td>
<td>C. albicans SC5314 Caf2:FR</td>
</tr>
</tbody>
</table>
BIOGRAPHY OF THE AUTHOR

Lena Stasiak was born in 1997, as Lena A. Stasiak, in Western Springs, Illinois, USA. She graduated from Whitefish Bay High School in Whitefish Bay, Wisconsin, in 2016. She is now anticipating graduation from the University of Maine with a Bachelor of Science degree in Zoology and a minor in Microbiology. While studying at the University of Maine, Lena was involved in Greek Life, and was appointed to the Secretary position on the Leadership Council of Alpha Omicron Pi, Gamma Chapter. Additionally, she studied abroad in Tanzania and conducted an independent research project on coral reef health in the Ushongo Village, Tanga Region. In 2018, she joined the lab of Dr. Clarissa Henry as an undergraduate research assistant to PhD student Elisabeth Kilroy. In 2019, she transferred to the lab of Dr. Robert Wheeler in order to complete her Honors Thesis to satisfy both the zoology major and microbiology minor. Lena was a recipient of the INBRE Thesis Fellowship for the 2019-2020 academic year, which helped to fund efforts for the thesis process.