HIGH SCHOOL EDITION
Journal of Student Research
Volume 13 Issue 1 (2024)

# Analysis of Zebrafish Innate Immunity Using Two Bacterial Species

Irene Jin[1], Eric Ju[1] and Sera Oh[#]

[1]Chapel Hill High School, USA
[#]Advisor

## ABSTRACT

Depending on the presence of an amniotic sac, which protects embryos from desiccation, vertebrates can be divided into two categories: amniotes, vertebrates with amniotic sacs, and anamniotes, vertebrates without amniotic sacs. As a result, anamniotes, which include the embryos of fish and amphibians, must develop in aquatic environments. In addition, due to the lack of an amniotic sac, which also serves as a barrier from the outside environment, anamniotes are more susceptible to bacterial infection at early stages than their amniotic counterparts. While it has been known that anamniotes possess immune systems similar to those of amniotes, it is still unclear how their immune systems fight against bacterial infection during early development. In this paper, I have examined how zebrafish embryos protect themselves from bacterial infection in order to understand the importance of the immune system during early development in anamniotes. My analysis shows that zebrafish embryos utilize phagocytic immune cells to suppress bacterial infection. Moreover, zebrafish embryos appear to be more vulnerable to infection by Gram negative than Gram positive bacteria, suggesting that the efficacy of phagocytic immune cells in anamniotes varies depending on the type of pathogen being fought against.

## Introduction

Amnion is a thick, impermeable membrane that sheaths embryos, protecting them from desiccation and bacterial infection during development (Starck et al., 2021). Depending on the presence or absence of the amnion, vertebrates can be categorized into two groups: amniotes and anamniotes. Mammals, birds, and reptiles belong to the first category, while fish and amphibians belong to the latter category. As amnion plays a critical role in protecting embryos from the environment, amniotic embryos are less susceptible to infection. However, most anamniotes need to develop in an aquatic environment to ensure that they are not dehydrated, increasing the risk of infection. Thus, it is essential for anamniotes to have a strong defense against bacteria, the most well-known method being the immune system, which is composed of diverse cell types, such as lymph nodes (Krishnamurty and Turley, 2020).

Immune cells are found in a diverse range of animals, including both vertebrates and invertebrates. Despite their diverse functions, the most important cellular activity of the immune cells is phagocytosis, the ingestion of foreign objects. Macrophages and neutrophils are the best known examples of phagocytic cells, appearing during early development as a part of innate immunity, an immediate immune response that does not distinguish the type of pathogen or foreign object introduced into the body (Uribe-Querol and Rosales, 2020). The efficacy of macrophages and neutrophils in eliminating foreign objects differs based on the surface properties of their targets. For example, when bacteria invade an organism, the ability of macrophages and neutrophils to eradicate infection depends on the cell wall structure of the bacteria. The cell wall, the outermost layer of bacterial cells, plays a critical role in protecting bacteria from the outer environment. Based on the composition of the cell wall, bacteria can be divided into two types: Gram positive bacteria and Gram negative bacteria. Gram positive bacteria possess a proteoglycan-based cell wall, while Gram negative bacteria possess an outer

membrane in lieu of a complex cell wall (Silhavy et al., 2010). As a result, due to the absence of an outer protectant, Gram positive bacteria are less resistant to phagocytic immune cells, making them easier to eliminate (Akira et al., 2006). In contrast, Gram negative bacteria are more resistant to both phagocytic cells and antibiotics, rendering them more potent pathogens (Akira et al., 2006; Silhavy et al., 2010). As such, amphibians and fish are generally more vulnerable to Gram negative bacterial infections. However, many Gram positive bacteria such as *Streptococcus* can cause severe infection in both amphibians and fish, and the mortality rates of these infections appear to be higher than those observed in Gram negative bacterial infections (Sirimanapong et al., 2018).

Thanks to their many unique attributes, zebrafish are the ideal candidate for observation of early development in anamniotes as well as the influence of bacterial infections (Gore et al., 2018). For instance, the immune system of zebrafish has been shown to retain similar physiological features as humans. Zebrafish embryos with identical genetic backgrounds can also be readily obtained by breeding, as one pair of zebrafish can produce 400 embryos per week in optimal conditions (Beis and Stainier, 2006). In addition, their smaller size allows them to be raised in a compact space. Moreover, zebrafish embryos are transparent, allowing for complex histological analyses. Finally, the zebrafish genome sequence has been completely annotated. Genetic manipulations are also relatively easier among vertebrates, allowing the findings obtained from research to be applied to other species, increasing the implications of the research. In this report, I aimed to understand how zebrafish embryos protect themselves from bacteria in their environment and fight against potential infections. In addition, by comparing the effects of Gram positive and Gram negative bacterial infections in zebrafish, I hoped to assess the influence of different bacterial cell walls on the outcomes of infection in zebrafish embryos.

## Hypotheses

I hypothesized that 1) zebrafish embryos may have an innate immune response to ward off the bacterial infection during early development, and 2) the efficacy of immune responses may be distinct between Gram positive and Gram negative bacteria, with Gram negative bacteria being more harmful to developing embryos.

## Statement of Purpose

Anamniotic embryos are exposed to ambient bacteria as they grow in aquatic environments. Thus, they must have an effective defense mechanism against bacterial infection. The purpose of this experiment is to elucidate how anamniotic embryos protect themselves from such threats during development to ensure their survival by using zebrafish (Danio rerio) as a model system.

## Materials

Wild type Danio rerio embryos
Sudan Black B
*Pseudomonas aeruginosa*
Pronase
Centrifuge
Phosphate-buffered saline (PBS pH 7.4)
12 well plates
Bacterial culture plate
Microscope with camera
*Staphylococcus epidermidis*
Embryonic media
Bacterial LB media
4% formaldehyde in PBS

# Methods

## Preparation of Zebrafish Embryos

Zebrafish embryos were obtained by mating wild type AB strain zebrafish, as previously reported (Sprague et al., 2006). Embryos were synchronized at 6 hours post-fertilization to ensure their developmental stages remained identical. Dead embryos and any debris were simultaneously removed to minimize bacterial infection. Embryos were raised in embryonic media, which contained 0.33mM of CaCl2•2H2O, 0.33mM of MgSO4•7H2O, 0.1% Methylene blue, and 10L of distilled water (Sprague et al., 2006). 50 U/mL of penicillin was also added to the media to inhibit bacterial growth, which was removed upon the introduction of bacteria to the media. At 24 hours post-fertilization, embryos were treated with Pronase to remove chorion (Sprague et al., 2006); embryos were briefly submerged in embryonic media containing Pronase for 30 minutes at room temperature. They were then washed five times to remove the residual Pronase. Subsequently, 10 zebrafish embryos were placed in each well of the 12 well plates, and bacterial suspension was introduced to the media, allowing infection to occur. For statistical analysis purposes, experiments were repeated at least five times.

## Preparation of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*

First, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were grown on the LB agar plates, and a single colony from each plate was used to inoculate 500mL of LB liquid media. The bacteria cultures were then incubated for 24 hours at 37 degrees and subjected to centrifugation to spin down bacteria. The resulting pellets were resuspended and washed five times with zebrafish embryo media to remove any residual LB liquid media. Finally, the bacterial cultures were resuspended in 0.5mL of zebrafish embryonic media. Serial dilution was then performed to prepare the inoculum for the zebrafish embryos, and 1:100, 1:1000, and 1:10000 dilutions were used to assess the effects of the bacteria on zebrafish mortality.

## Assessing Response Toward the Bacteria Infection

To see if the introduction of the bacteria to the media would increase the number of innate immune cells in zebrafish embryos, the embryos were fixed with 4% formaldehyde in PBS overnight and later washed five times with PBS. Then, fixed zebrafish embryos were incubated in Sudan Black B solution in the dark for 1 hour at room temperature. They were subsequently washed with PBS multiple times to remove excessive staining solution. The tailmost region of the zebrafish embryos was analyzed with a stereo microscope. Each embryo was photographed, and the resulting images were analyzed to quantify the number of Sudan B positive cells within zebrafish embryos, or the number of innate immune cells.

## Deducing Bacteria-Induced Mortality in Zebrafish Embryos

The mortality of zebrafish embryos was assessed through observation under stereomicroscopes, as live zebrafish embryos continuously twitch and show stereotypic foraging behaviors (Sprague et al., 2006). The number of live embryos was counted every 24 hours, and the dead embryos were removed by capillary pipette to minimize the bacterial expansion. The mortality rate was then calculated as the number of total deaths divided by the number of total individuals, later converted as a percentage for comparison.

## Results

### Zebrafish Embryos Have Phagocytic Immune Cells During Development

As previously mentioned, I examined the presence of cells stained with Sudan Black B in order to determine whether zebrafish embryos have innate immune cells during early development. Sudan Black B stains the lipophilic granules within cells, which are rich in phagocytic immune cells (Diaz-Satizabal and Magor, 2015). Zebrafish embryos appear to have a significant number of cells stained with Sudan Black B at 2dpf (Figure 1A). On average, I found that 2dpf zebrafish embryos contain 43.2±12.54 Sudan Black B stained cells (Figure 1B). Considering the location and shape of the cells, these stained cells appear to be immune cells, with the majority of them residing within blood vessels. The number of phagocytic immune cells in zebrafish embryos continued to increase up to 4dpf, at which point the average number of Sudan Black B stained cells per embryo reached 76.5±11.8. However, the number of Sudan Black B stained cells was slightly reduced in 5dpdf zebrafish embryos, although there was no significant difference between the number of Sudan Black B stained cells in 4dpf and 5dpf embryos. Considering that immune cells are formed in the caudal hematopoietic tissue (CHT) (Murayama et al., 2006) and migrate out to spread over the body, it appears that immune cells began to migrate out of this region at 5pdf.

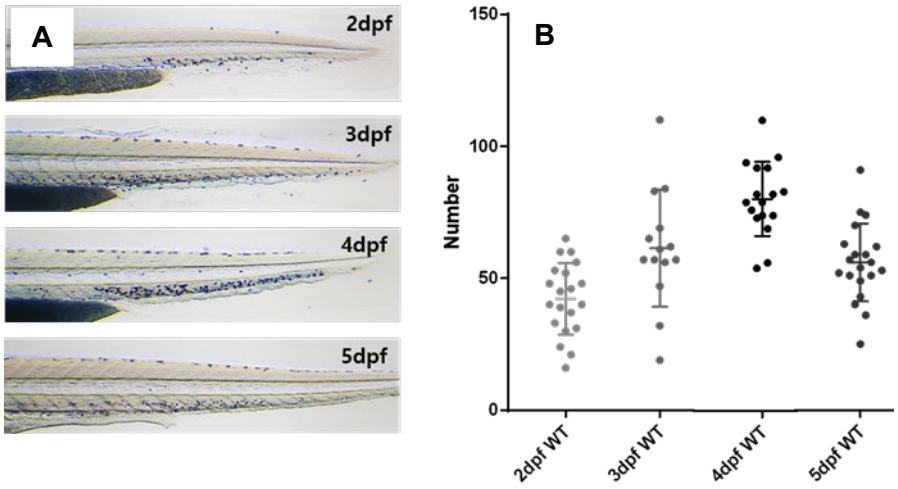


**Figure 1**. Zebrafish larvae have Sudan Black B stained immune cells. The photomicrographs show the presence of Sudan Black B stained cells in the tailmost region of developing zebrafish embryos (A) and the quantification of Sudan Black B stained cells per embryo (B). Zebrafish embryos were stained with Sudan Black B and the tailmost region of the embryos was photographed under the stereomicroscope for further quantification. As Sudan Black B preferentially stains immune cells with granules, the number of those cells can be deduced by counting the number of Sudan Black B stained cells. The number of Sudan Black B stained cells in zebrafish embryos within the tailmost region was counted and quantified. The number of Sudan Black B stained cells continuously increased up to 4 days post-fertilization. At 5 days post-fertilization, the number of Sudan Black B stained cells decreased as the immune cells infiltrated into the circulation by that time.

### Zebrafish Embryos Increase the Number of Phagocytic Immune Cells Upon Infection

As shown in Figure 1, zebrafish embryos had a significant number of cells stained with Sudan Black B prior to the infection, showing that phagocytic immune cells are present during early development in zebrafish embryos

(Diaz-Satizabal and Magor, 2015; Gore et al., 2018). Considering that Sudan Black B stains both macrophages and neutrophils, it is possible that the stained cells could be either. However, given the fact that macrophages are the first immune cell to develop in zebrafish embryos (Gore et al., 2018), it is likely that these cells are macrophages. Thus, it appears that zebrafish embryos do possess innate immune cells in early development.

After infection, the number of Sudan Black B stained cells significantly increased, suggesting that zebrafish embryos respond to bacterial infection by increasing the number of phagocytic immune cells (Figure 2A). Mock-infected zebrafish embryos possessed an average of 189.57±45.74 Sudan Black B stained phagocytic immune cells. However, upon infection with Gram negative bacteria, *Pseudomonas aeruginosa*, the number of Sudan Black B stained cells rose to an average of 235.60±22.12 per embryo. In comparison, upon infection with Gram positive bacteria, *Staphylococcus epidermidis*, the number of phagocytic immune cells rose to an average of 250.66±23.92, surpassing the average of embryos infected with Gram negative bacteria. Statistical analysis showed that the number of phagocytic immune cells in zebrafish embryos exposed to either bacterium was statistically different from that of the control (p value=0.0429 for *Pseudomonas aeruginosa* exposed zebrafish embryos, and p value=0.0219 for Staphylococcus epidermidis exposed zebrafish embryos) (Figure 2B). While not statistically significant (p value=0.35), it appears that there is a tendency for *Staphylococcus epidermidis* to be more immunogenic than *Pseudomonas aeruginosa* - in other words, there is a tendency for Gram positive bacteria to be more immunogenic than Gram negative bacteria.

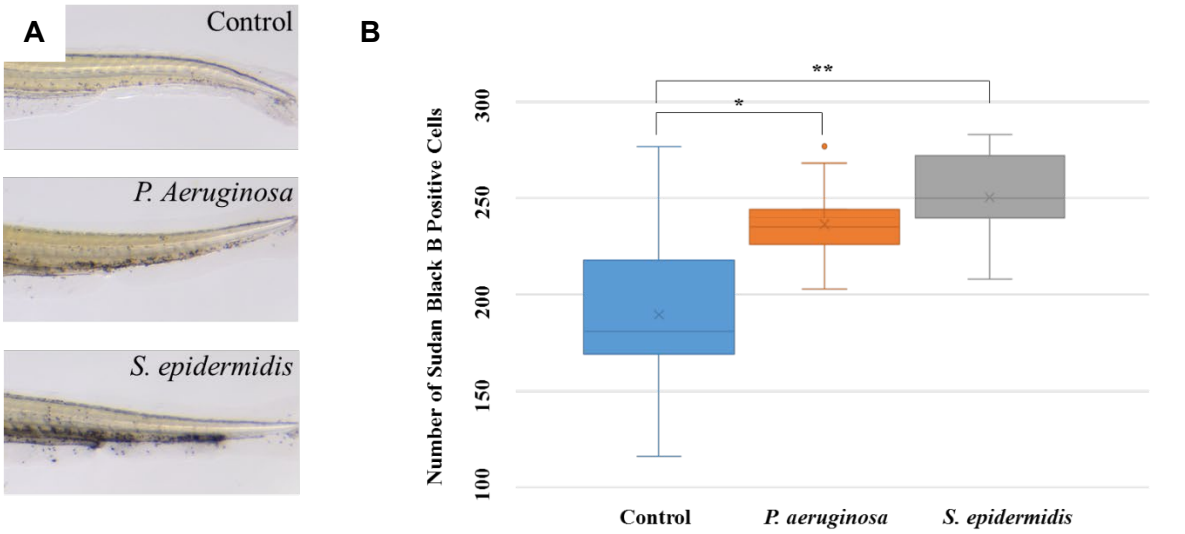


**Figure 2**. Presence of Sudan Black positive phagocytic cells in zebrafish embryos. The photomicrographs show the presence of Sudan Black B stained cells in the tailmost region of developing zebrafish embryos exposed to two bacteria species, compared to mock-infected control (A), and the quantification of Sudan Black B stained cells per embryo (B). The number of Sudan Black B stained cells in zebrafish embryos within the tailmost region was significantly increased upon infection with either *Pseudomonas aeruginosa* or *Staphylococcus epidermidis*. While it appears that infection with *Staphylococcus epidermidis* elicits more robust immunogenic responses and acts as a more potent booster for immune cells than infection with *Pseudomonas aeruginosa*, it was not statistically significantly different.

## Gram Positive and Gram Negative Bacteria Distinctly Increase the Mortality Of Zebrafish Embryos

To measure the effects of bacterial infection on anamniotes and the efficacy of the zebrafish's innate immune system, hatched zebrafish embryos were exposed to different concentrations of *Pseudomonas aeruginosa* or *Staphylococcus epidermidis*. As shown in Figure 2, the mortality rate of zebrafish embryos significantly increased in those exposed to the bacteria than in those who were not exposed, or the control. To examine if the presence of a bacterial wall influenced the outcomes of bacterial infection in zebrafish embryos, I examined the effects of *Pseudomonas aeruginosa* as a representative of Gram negative bacteria and *Staphylococcus epidermidis* as a representative of Gram positive bacteria. By comparing the two, I found that zebrafish exposed to *Staphylococcus epidermidis* had a higher mortality rate than those exposed to *Pseudomonas aeruginosa*. At 1.5 days post-fertilization, there was no difference among zebrafish embryos in terms of mortality rate. However, those exposed to bacteria showed a rapid increase in mortality rate compared to the control group. For instance, at 2 days post-fertilization, the mortality rate of zebrafish embryos exposed to *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* was significantly higher than the control group (4.16±0.7 percent for *Pseudomonas aeruginosa* exposed zebrafish embryos and 5.21±1.05 for *Staphylococcus epidermidis* exposed zebrafish embryos, with a p value of 0.0417 and 0.082 respectively). At 2.5 days post-fertilization, the differences in mortality rate became even more apparent, and at 3.5 days post-fertilization, the mortality rate of the control group remained as low as 3.1±1.1 percent. In comparison, those exposed to *Pseudomonas aeruginosa* had a 22.9±2.66 percent mortality rate and those exposed to *Staphylococcus epidermidis* had a 28.1±3.17 percent mortality rate at 3.5 days post-fertilization, with both having differing statistical significance (p value=0.0067 for *Pseudomonas aeruginosa* exposed zebrafish embryos and p value=0.0037 for *Staphylococcus epidermidis* exposed zebrafish embryos). Although it was not statistically significant (p value=0.5593 with 95% confidence), the tendency for *Staphylococcus epidermidis* to be more harmful than *Pseudomonas aeruginosa* was seen.

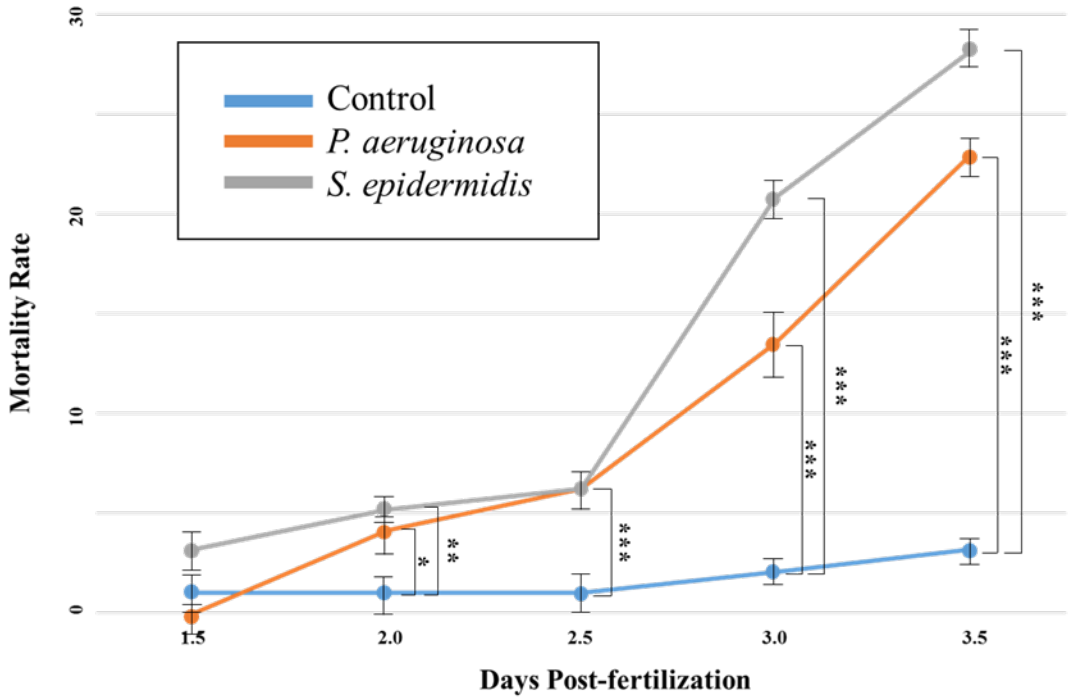


**Figure 3**. Effects of Gram positive and Gram negative bacteria on the mortality of zebrafish embryos. The mortality of zebrafish embryos exposed to *Staphylococcus epidermidis* or *Pseudomonas aeruginosa* was calculated and compared with mock-infected zebrafish embryos. Both Staphylococcus *epidermidis* or *Pseudomonas*

*aeruginosa* infected zebrafish embryos show a significantly elevated mortality rate compared to the mock-infected control group.

## Discussion

The initial hypothesis, which proposed that zebrafish embryos have a functional immune system in early developmental stages, appears to be supported by the data provided above, preventing its rejection. Meanwhile, the second hypothesis, which proposed that the efficacy of innate immune responses may be distinct between Gram positive and Gram negative bacteria, with Gram negative bacteria being more resistant, may need to be examined further. Though the differing mortality rates provided in the data suggest that the potency of immune responses varies depending on the presence of a cell wall, I was surprised to find that *Staphylococcus epidermidis*, a Gram positive bacteria, had a higher mortality rate than *Pseudomonas aeruginosa*, a Gram negative bacteria. As a result, the second hypothesis must be rejected. However, this could be a premature conclusion, as only one species of Gram positive and Gram negative bacteria was used in the experiment. While my data clearly showed that *Staphylococcus epidermidis* is a more potent pathogen than *Pseudomonas aeruginosa*, these results may simply reflect the differences between these two species rather than being a whole representation of the differences between Gram positive and Gram negative bacteria. Thus, further experimentation is warranted to form a comprehensive understanding of the differences in pathogenicity between these two bacterial types.

My findings suggest that zebrafish can ward off pathogens even early in development. Since zebrafish is a widely used aquatic model due to its evolutionary conservation and physiological similarity to other species, it is plausible that other aquatic anamniotes use similar mechanisms to fight against bacterial infection early on to ensure proper development. In addition, my results suggest that bacterial infection is most likely detrimental for aquatic animals during early development. While the majority of the embryos survived, a sizable portion of the zebrafish embryos succumbed to the bacterial infection. As the production of farm-raised fish has continued to increase, minimizing bacterial infection during the embryonic stage is increasingly critical. Consequently, the long-term health effects of an increased use of antibiotics in the commercial aquaculture industry have become a subject of discussion (Hegde et al., 2023). While it is imperative that excessive usage of antibiotics be restricted, my findings also indicate that the proper usage of antibiotics could be highly beneficial, protecting the fish at their most vulnerable stages of development and thus increasing productivity and profitability. Similarly, the reduction of organic pollutants, and by extension, the environmental concentration of bacteria, could help sustain endangered marine organisms.

## Conclusion

I have shown that zebrafish embryos possess phagocytic immune cells during early development, as zebrafish embryos at 2 days post-fertilization contain a significant number of cells stained with Sudan Black B, which recognizes granules within phagocytic immune cells. In addition, I have shown that exposure to bacteria increases the number of these immune cells as a defense mechanism, suggesting the existence of robust immune responses at early developmental stages. Having phagocytic immune cells at an early developmental stage may help anamniotic embryos fight against the infection.

## Limitations and Future Research

As only two species of bacteria have been analyzed, it is imperative that more bacterial species be examined in order to fully support my second hypothesis: Gram positive bacteria is less harmful than Gram negative bacteria

to developing zebrafish embryos. Moreover, considering that zebrafish have a number of different strains, each with distinct genetic backgrounds (Beis and Stainier, 2006; Sprague et al., 2006), it is necessary to include different strains of zebrafish for more accurate assessments on the effects of bacterial exposure to minimize the innate genetic variation among strains.

## Acknowledgments

I would like to thank Gwangju Institute of Science and Technology for a summer internship opportunity and Ms. Sera Oh for guidance and her help in experimental design for summer internship.

## References

Akira, S., Uematsu, S. and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell* 124, 783-801.

Beis, D. and Stainier, D. Y. (2006). In vivo cell biology: following the zebrafish trend. *Trends Cell Biol* 16, 105-112.

Diaz-Satizabal, L. and Magor, B. G. (2015). Isolation and cytochemical characterization of melanomacrophages and melanomacrophage clusters from goldfish (Carassius auratus, L.). *Dev Comp Immunol* 48, 221-228.

Gore, A. V., Pillay, L. M., Venero Galanternik, M. and Weinstein, B. M. (2018). The zebrafish: A fintastic model for hematopoietic development and disease. *Wiley Interdiscip Rev Dev Biol* 7, e312.

Hegde, A., Kabra, S., Basawa, R. M., Khile, D. A., Abbu, R. U. F., Thomas, N. A., Manickam, N. B. and Raval, R. (2023). Bacterial diseases in marine fish species: current trends and future prospects in disease management. *World J Microbiol Biotechnol* 39, 317.

Krishnamurty, A. T. and Turley, S. J. (2020). Lymph node stromal cells: cartographers of the immune system. *Nat Immunol* 21, 369-380.

Murayama, E., Kissa, K., Zapata, A., Mordelet, E., Briolat, V., Lin, H. F., Handin, R. I. and Herbomel, P. (2006). Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity* 25, 963-975.

Silhavy, T. J., Kahne, D. and Walker, S. (2010). The bacterial cell envelope. *Cold Spring Harb Perspect Biol* 2, a000414.

Sirimanapong, W., Thompson, K. D., Shinn, A. P., Adams, A. and Withyachumnarnkul, B. (2018). Streptococcus agalactiae infection kills red tilapia with chronic Francisella noatunensis infection more rapidly than the fish without the infection. *Fish Shellfish Immunol* 81, 221-232.

Sprague, J., Bayraktaroglu, L., Clements, D., Conlin, T., Fashena, D., Frazer, K., Haendel, M., Howe, D. G., Mani, P., Ramachandran, S., et al. (2006). The Zebrafish Information Network: the zebrafish model organism database. *Nucleic Acids Res* 34, D581-585.

Starck, J. M., Stewart, J. R. and Blackburn, D. G. (2021). Phylogeny and evolutionary history of the amniote egg. *J Morphol* 282, 1080-1122.

Uribe-Querol, E. and Rosales, C. (2020). Phagocytosis: Our Current Understanding of a Universal Biological Process. *Front Immunol* 11, 1066.