Examination of the effects of fever and anti-pyrectics on goldfish thrombocytes

by

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ABSTRACT

Ancient philosophers observed and documented fever as an increased body temperature in response to infection. Nowadays, fever is regarded as a symptom of infection and inflammation and therefore is often supressed by NSAID drugs. A common hypothesis for the benefits of a fever is that the rise in temperature hinders a pathogen's virulence and acts as a switch to activate the body's immune response. However, in literature, there is ongoing debate about the exact effect of fever on host health and host response to immune challenge, therefore, begging the question: does fever have net-positive or net-negative effects on host health? To answer this question, early studies utilized endothermic models placed in higher or lower temperature environments than their average core body temperature. This however, added the confound heat induced stress responses which also impacted host health and immune responses. Interestingly, fever has been conserved for millions of years, and in ectothermic species, a raise in core body temperature is achieved by relocation to warmer environments. Ectotherms such as reptiles, amphibians, fish and insects display behavioral thermoregulation. The conservation of febrile responses spans over more than 550 million years of evolution, further procuring the question about the potential benefits of fever for host health.

Previous studies using teleost models have shown that behavioral fever can upregulate pro-inflammatory and anti-inflammatory gene expression at different timepoints in acute inflammation. Our lab has previously characterized enhanced immune responses in febrile conditions showing faster recruitment kinetics, altered anti-microbial profiles of effector cells and a more efficient switch from pro-inflammation to pro-resolution. While some leukocyte subsets have been examined, no teleost studies have shown the impact of fever on thrombocytes. Thrombocytes are considered "ancient platelets" and platelets in mammals have been shown to

play critical roles in immune responses and possess immunomodulator capacities. While platelets were first thought to only have a role in hemostasis, they are now regarded as a dynamic cell population with versatile interactions and functions throughout the body. Thrombocytes have been observed in many ectothermic species including amphibians, reptiles, avians and fish with a high degree of conservation in cell morphology. However, aside from morphology and some studies on functional capacities of goldfish thrombocytes, little is known about thrombocyte functions within immune responses. Furthermore, while some studies have highlighted conserved functions in thrombocytes, the quantification of thrombocytes participating in the site of immune challenge is limited. While mammalian studies have long demonstrated that NSAIDs like aspirin impact platelet function, the impact of NSAID induced fever-inhibition on thrombocytes has not been explored. To examine the impact of fever-inhibition I used an established cutaneous infection model for tissue repair. Tissue repair is a tightly regulated process that relies on stages such as inflammation for pathogen clearance after tissue injury. Platelets have been long demonstrated to be crucial in tissue repair and therefore the quantification of thrombocytes in febrile and fever-inhibited conditions may highlight a potential benefit of a febrile response.

Overall, my results show that fever alters the kinetics of leukocytes and thrombocytes in the peritoneum in both a natural fever and fixed temperature conditions. Febrile responses were associated with an earlier recruitment of leukocytes consisting primarily of neutrophils, followed by macrophages. Leukocytes in febrile conditions demonstrated a significant reduction in ROS and increase in NO for pathogen clearance. Furthermore, lymphocyte involvement was increased in natural fever conditions. Thrombocyte populations and proliferation was significantly reduced in the peritoneum of fish in febrile conditions compared to fixed temperature conditions. The

disparity in thrombocyte population was even greater in the tissue repair model between febrile and fever-inhibited conditions. Furthermore, thrombocyte upregulation was sustained in fever-inhibited conditions compared to febrile conditions, and key pro-inflammatory and anti-inflammatory cytokines involved in tissue repair were dysregulated in fever-inhibited conditions. Finally, histological staining revealed the delay in tissue repair in fever-inhibited conditions. Taken together the data suggests a dysregulation of acute inflammatory processes by ketorolac fever-inhibition.

PREFACE

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Some of the research conducted during my thesis have been apart of collaborations, published (or are in the process of publication) in peer-reviewed journals as follows:

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- Michnik ML, Semple SL, **Joshi RN**, Whittaker P., Barreda D.R. The use of salmonid epithelial cells to characterize the toxicity of *Tenacibaculum maritimum*-soluble extracellular products. Journal of Applied Microbiology.: lxae049. doi:https://doi.org/10.1093/jambio/lxae049. https://pubmed.ncbi.nlm.nih.gov/38409849/.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

2-AG 2-arachidonicglycerol

AA arachidonic acid

ACP acid phosphatase

ADP adenosine diphosphate

ANEA A-napthyl acetate esterase

APC antigen presenting cells

ATPT annular thermal preference tank

CCL2 chemokine (C-C motif) ligand 2

CCL7 chemokine (C-C motif) ligand 7

c-mpl thrombopoietin receptor

COX2 cyclooxygenase 2

CSF-1R colony stimulating factor receptor 1

CSR class switch recombination

CXCL8 Chemokine (C-X-C motif) ligand 8

DAMP damage associated molecular pattern

DNA deoxyribonucleic acid

dpi says post infection

ECM extracellular matrix

EGF epidermal growth factor

ETC electron transport chain

FGF fibroblast growth factor

G-CSF granulocyte colony stimulating factor

HMGB1 high mobility group box 1 protein

hpi hours post injection

HSP heat shock protein

IFNγ interferon gamma

Ig immunoglubulin

IL-1 Interleukin 1

IL-10 interleukin 10

IL10R1 interleukin 10 receptor subunit 1

IL10R2 interleukin 10 receptor subunit 2

IL1β Interleukin 1 beta

IL-6 Interleukin 6

IL-8 Interleukin 8

IM intramuscular

iNOS inducible nitric oxide synthase enzyme

IV intravenous

JAK Janus kinase

LPS lipopolysaccharide

mAb monoclonal antibody

MAF macrophage activating factors

MCSF-R macrophage colony stimulating factor receptor

MGF macrophage growth factor

MMR macrophage mannose receptor

MPO myeloperoxidase

NET neutrophil extracellular trap

NFκB nuclear factor kappa-light-chain-enhancer of activated B cells

NK natural killer

NO nitric oxide

NSAID non steroidal anti-inflammatory drugs

PAF platelet activating factor

PAMP pathogen associated molecular pattern

PAS periodic acid schiff

PCR polymerase chain reaction

PGE2 Prostaglandin E2

PKM primary kidney macrophages

PLA2 phospholipase A 2

PRR pattern recognition receptor

qPCR quantitative polymerase chain reaction

RNA ribonucleic acid

RNI reactive nitrogen intermediates

ROS reactive oxygen species

SOCS3 supressor of cytokine signalling 3

STAT3 signal transducer and activator of transcription

SYK spleen tyrosine kinase

T1R type 1 taste receptor

T2R type 2 taste receptor

TCR T cell receptor

TD dynamic temperature

TGFβ transforming growth factor beta

Tket ketorolac conditions

TLR toll-like receptor

TLR2 toll-like receptor 2

TLR6 toll like receptor 6

TMS tricane mesylate

TNF Tumor necrosis factor

TNFR-1 tumor necrosis factor receptor 1

TNFR-2 tumor necrosis factor receptor 2

TNFα Tumor necrosis factor alpha

TS static temperature

VEGF vascular endothelial growth factor

v-mpl myeloproliferative leukemia virus oncogene

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Fever is the physiological increase in the core body temperature of an organism, often in response to an infection. Fever has been conserved through million years of evolution. The earliest definitions of fever arise from ancient Greek physicians such as Hippocrates in 400 BC and Galen 200 AD, in which a systemic increase in temperature was qualitatively observed. Furthermore, these ancient physicians categorized various fevers, indicating that patients often did not recover from ongoing high fevers, but a short febrile period allowed for enhanced healing capacity (Sajadi et al. 2012). Therefore, the debate of if fever had a net-negative or net-positive effect was ongoing. To complicate matters even further, in efforts to replicate a physiological fever, several models increase external temperature of an endothermic animal. This in turn causes physiological stress, resulting in systemic changes throughout the body, which cannot be directly correlated to having a fever.

Fever in endotherms is initiated through the detection of damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on myeloid cells which triggers a cascade of molecular pathways that make up the pro-inflammatory response (Evans et al. 2015). Innate immune cells that detect PAMPs will release prostaglandin E_2 (PGE2) and pyrogenic cytokines (cytokines that can initiate the febrile response) such as Interleukin-1 (IL-1), Interleukin-6 (IL-6) and tumor necrosis factor (TNF). IL-6 acts on the median preoptic nucleus region of the hypothalamus and stimulates the synthesis of cyclooxygenase 2 (COX2), an enzyme that generates additional PGE_2 (Coceani et al. 1986). This prostaglandin will bind to EP3 receptors and trigger the sympathetic nervous system to release noradrenaline. Noradrenaline will increase thermogenesis in brown adipose tissue and induce vasoconstriction to reduce passive heat loss (Evans et al. 2015). Up until the 1970s it was thought that only endotherms had fevers, however, in the 1970s several research groups reported that ectothermic animals displayed a behavioral relocation to warmer temperature environments to increase core body temperature (Reynolds 1977; Kluger 1979). Soon after, it was discovered that both endotherms and ectotherms utilize PGE_2 and their central nervous systems to induce febrile responses (Evans et al. 2015). Since then, behavioral thermoregulation by ectotherms has been a powerful tool in examining the febrile response as ectotherms re-locate to warmer

temperatures, thereby bypassing the thermal stress effects caused by increasing environmental temperatures in endotherms.

Fever is a hallmark of acute inflammation, and concurrent with studies exploring the conservation of fever, several other aspects of acute inflammation are also conserved throughout evolution (Hurley 1972; Evans et al. 2015). The numerous examples of the conservation of the components of acute inflammation point to acute inflammation being a necessary process in host defense against the external environment to restore homeostasis. A fundamental pathway in the restoration of homeostasis is the tissue repair pathway. The tissue repair pathway is a complex biological process that involves interaction between connective cells at the site of tissue injury and various populations of immune cells. It consists of four tightly regulated phases including: hemostasis, inflammation, proliferation, and tissue remodeling (Gonzalez et al. 2016; Schultz et al. 2011). Upon detection of tissue injury, pathways to reduce blood loss are activated, and a hemostatic plug is formed via platelet and erythrocyte interaction (Periayah et al. 2017). Several leukocytes are recruited by activation of hemostasis pathways to clear any pathogens that entered the host. Concurrently, there is an increase in several pro-inflammatory cytokines and chemokines such as IL1β, TNFα, and IL-8 for the recruitment of leukocytes and promotion of acute inflammation (Chen et al. 2018). Recruited innate immune cells carry out various anti-microbial effector functions such as pathogen capture, phagocytosis, and release of antimicrobial molecules such as reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) (Underhill et al. 2005). After pathogen clearance, several other cytokines are upregulated for the resolution of acute inflammation including IL-10, and TGF-β (Iyer and Cheng 2012; Chen et al. 2018). The resolution of acute inflammation leads to the initiation of the proliferative phase of tissue repair in which leukocytes such as macrophages, neutrophils and platelets release several growth factors such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) (Schultz et al. 2011). The upregulation of growth factor release is supported by cytokines such as IL-10 and TGFβ, and the proliferation of epidermal cells, vascular cells and fibroblasts results in the rebuilding of damaged tissue. The proliferating cells in turn create various components of tissue structure such as collagen (Gonzalez et al. 2016).

In this thesis, I utilized a teleost fish model to examine the contributions of fever in a fundamental process of restoring homeostasis, tissue repair. Furthermore, I examined the impact of fever inhibition on a dynamic but under-characterized cell population, thrombocytes (platelet ancestor). With the use of a self resolving zymosan induction of acute inflammation, I was able to identify and enumerate thrombocytes within the peritoneum throughout the acute inflammatory window. With the use of a self resolving cutaneous infection with *Aeromonas veronii*, I was able to examine impact of and anti-pyretic (fever inhibiting drug) ketorolac on tissue repair. Over the years my lab has contributed various investigations that exemplify the immune enhancing feature of a natural febrile response. I hypothesized that the inhibition of fever would subdue the immune enhancement effect of fever, and lead to pathophysiological states in tissue repair.

1.2 THESIS OBJECTIVES

The main objective of my thesis was to characterize the potential contributions of fever in acute inflammation by assessing the impact of fever on leukocytes and thrombocytes after immune challenge. The two immune challenge models I utilized to represent restoration of homeostasis were a self resolving zymosan induced peritonitis, and a cutaneous epidermal infection with a gram-negative bacterium. To dissect the impact of fever on leukocyte and thrombocyte kinetics, fish after immune challenge were placed in either fixed static temperature tanks or allowed to exhibit thermal preference in a dynamic temperature gradient (ATPT). My specific cell of focus was thrombocytes, often referred to as ancient platelets. My research focussed on assessing the contributions of a dynamic behavioral fever on thrombocyte kinetics and restoration of homeostasis in tissue repair. Specific aims were (1) characterization of goldfish thrombocytes using histology, flow cytometry and qPCR, (2) assessing thrombocyte kinetics in static and dynamic temperature conditions, (3) assessing the impact of fever-inhibition on thrombocytes in tissue repair, and (4) assessing the impact of fever-inhibition on overall tissue repair.

1.3 ACUTE INFLAMMATION AND FEVER

1.3.1 Acute inflammation

Inflammation is a conserved process in vertebrate immunity that is tightly regulated using an array of cellular and protein cascades, which occurs in response to tissue injury and/or pathogenic entry into the host organism. The five cardinal signs of inflammation were

characterized by ancient Roman philosophers between 30 B.C. and 200 A.D., which are: *rubor* (redness), *tumour* (swelling), *calor* (heat), *dolor* (pain), and *functio laesa* (loss of function) (Hurley 1972). Although these signs of acute inflammation are currently viewed as symptoms that require treatment, the requirement of inflammation in tissue healing and restoration of homeostasis have been known since the 19th century contributions of Metchnikoff (Medzhitov 2010). The duration of the upregulation of pro-inflammatory mediators and subsequent effector cells allows us to categorize inflammation as acute or chronic. Acute inflammation is observed to have a rapid onset and a short-lived duration lasting from days to weeks while chronic inflammation can have either a slow or rapid onset and a longer duration lasting from several months to years. The goal of acute inflammation is pathogen clearance, promotion of tissue repair and return to homeostasis (John Victor Hurley 1972; Medzhitov 2010; Tauber 2003). When acute inflammation is not resolved at a particular site, it becomes chronic inflammation and pathophysiological phenotypes become prevalent (Tauber 2003; Nathan and Ding 2010). Multiple cellular and molecular components in the inflammation timeline can trigger a switch to resolving the inflammation, such that it does not become chronic (Sugimoto et al. 2016). These interacting multi-level pathways can be amalgamated into the following phases: induction (using endogenous or exogenous pyrogens), cellular and protein mediation, transition to resolution of inflammation, resolution, and finally return to homeostasis (Serhan et al. 2020).

1.3.2 Fever

Fever, or pyrexia in endotherms is defined as an increase in core body temperature from basal temperatures that are predetermined by the thermoregulatory center in the organism's hypothalamus (Balli et al. 2022). For example, in humans, a low-grade fever is between 37.3-38.0℃, a moderate grade fever is between 38.1-39℃ and a high-grade fever is between 39.1-41℃ (Balli et al. 2022). There are multiple factors that determine an organism's core body temperature such as metabolic rate, body mass, and several ecological factors (Clarke and Rothery 2007). Interestingly fever has been conserved for over 550 million years in both endothermic and exothermic vertebrates (Kluger 1979). Endothermic organisms internally produce heat to elevate core body temperature, whereas endotherms rely on the environment to elevate core body temperature(Labocha and Hayes 2008). Endothermic regulation of body temperature can occur through generation of mechanical heat via shivering and evaporative cooling or transpiration (Balli et al. 2022). In addition to behavioral fever, endotherms utilize

metabolic processes that are altered in febrile conditions for thermogenesis, and prevention of additional heat loss (Terrien et al. 2011; Garami et al. 2018). Endothermic fever is classically initiated after the detection of DAMPs or PAMPs by PRRs on circulating or tissue resident cells that trigger a pro-inflammatory and antimicrobial response. Cytokines such as TNFα and IL1β act as potent pyrogenic cytokines that reach the pre-optic nucleus of the hypothalamus and induce an increase in prostaglandins such as PGE2. PGE2 plays a significant role in the nervous system resulting in vasoconstriction of peripheral blood vessels (to reduce heat loss), shivering of large muscles and thermogenesis (Roth and Blatteis 2014). In comparison, ectothermic vertebrates include fishes, amphibians and reptiles that physically relocate to warmer areas to cause an increase in body temperature (Evans et al. 2015; Kluger 1979). While fever is well known to be associated as a response to infection, there are still limited studies that examine the contributions of fever to host defence and survivability. Furthermore, to induce an increase in core body temperature, a fish swims to warmer waters often closer to the surface of a water body or the shore of a water body. In swimming to warmer waters, the fish increases its risk of predation and capture (Rakus et al. 2017). However, regulation of body temperature and induction of behavioral fever has been shown to enhance immune response to pathogens in multiple ectothermic organisms (Rakus et al. 2017; Evans et al 2015). Although fever is associated with potential benefits to host defense, an uncontrolled increase in temperature, termed hyperpyrexia, is implicated in multiple pathologies and mortalities in organisms (Balli et al. 2022; Walter et al. 2016). This has led to the development and administration of anti-pyretic drugs for the reduction of fever (Prajitha et al. 2019). However, the widespread use of anti-pyrectics throughout clinical and household settings is not sufficiently supported by scientific studies and potential benefits of thermoregulation may be attenuated in the suppression of fever (El-Radhi 2012). This may be because mammalian studies often utilize an increase in exogenous temperature to simulate a fever. However, exogenous mechanical temperature increases elicit physiological stress responses in endotherms and therefore fails to replicate an organism's internal thermoregulatory machinery.

1.4 CARASSIUS AURATUS AS A MODEL ORGANISM

Fever was first characterized in goldfish in 1977 in which the fever provided goldfish with a specific survival advantage (Covert and Reynolds 1977). After the initial characterization of fever in goldfish came the identification of fever in other teleosts (Bly and Clem 1992;Flajnik

1996;Köllner and Kotterba 2002). Unlike endotherms, ectotherms such as goldfish lack the certain metabolic pathways utilized by endotherms for generating or dissipating bodily heat, therefore the behavioral thermal preference of goldfish is a strong indicator of the febrile response (Kluger 1979; Hasday et al. 2014). Even amongst teleost, many species vary in their tolerated thermal range. Goldfish have a thermal tolerance from 0℃ to 43℃ and can be acclimated in water temperatures anywhere between 15℃ and 25℃ (Scharsack and Franke 2022). Due to the high range of thermal tolerance, goldfish are an ideal organism to examine fever without the risk of thermal stress.

1.5 INDUCTION OF THE FEBRILE RESPONSE

1.5.1 Aeromonas Veronii

1.5.1.1 Overview of the Aeromonas genus

Species within the Aeromonas genus are ubiquitously present and known to cause disease in many aquatic and mammalian species (Janda and Abbott 2010). During the mid-1970s, gram-negative aeromonads were organized into two major groups separated by growth characteristics and biochemical features (Fernández-Bravo and Figueras 2020; Janda and Abbott 201). A mesophilic group contained motile species with an optimal growth range between 35-37℃, and a psychrophilic group containing non-motile species with an optimal growth range between 22-25℃. Some species within the mesophilic group are associated with infections in humans while some species in the psychrophilic group caused diseases in fish and other aquatic animals (Janda and Duffey 1988; Joseph and Carnahan 1994). Using 16sRNA gene sequencing, multiple species and strains within the genus have been characterized, and phylogenetic studies continue to provide evidence for the biochemical distinctiveness between species and strains (Janda and Abbott 2010).

Aeromonas veronii was first distinguished from other members in the genus in 1988 and is a genomospecies with *Aeromonas hydrophilia* and *Aeromonas caviae* (Janda and Abbott 2010). These three genomospecies are responsible for greater than 85% of human infections and environmental isolations (Janda and Abbott 1998). Total bacterial isolation from infection and freshwater shows that *A. salmonicida* is more prevalent in infection of vertebrates and in freshwater, and *A. veronii* is more prevalent in vertebrates and invertebrates such as arthropods, as well as in both fresh and saltwater environments (Janda and Abbott 2010).

1.5.1.2 Mechanisms of virulence in Aeromonas

The complex virulence factors of Aeromonads contribute to immune evasion and infection of the host. Numerous virulence genes are associated with the pathogenicity of *Aeromonas* including aerolysin *(aer)* and hemolysin *(haem),* which aid in extracellular secretions, heat liable and head stable cytotoxic genes (*alt, ast)*, polar flagellum (*fla)*, lateral flagellum (*laf*), and others (Fujii et al. 2003; Bernheimer and Avigad 1974; Kirov et al. 2002; Tomás 2012;Beaz-Hidalgo and Figueras 2013). Additionally, *Aeromonads* utilize four secretion systems to excrete cellular products into the environment and host. The extracellular products aerolysin and hemolysin are potent hemolytic agents that aid *Aeromonas* in disrupting host membranes. The presence of both polar and lateral flagella aid in bacterial adhesion to host and overall motility in the environment, and both heat-liable and heat-stable cytotonic enterotoxin play immunomodulatory roles in the host's immune system (Sha et al. 2002 Tomás 2012;Beaz-Hidalgo and Figueras 2013). These common mechanisms are prevalent in most of the *Aeromonas* genus and aid in the overall virulence of *Aeromonas'*.

1.5.1.3 Aeromonas infections in fish

Being opportunistic pathogens, *Aeromonas* infection breakouts in freshwater and saltwater settings often occur in response to environmental changes (Chaix et al. 2017). In aquatic species, *Aeromonas* infection occurs through direct contact with the infected animal. Members of both the mesophilic and psychrophilic group can cause infections (Janda and Duffey 1988) (Søren Saxmose Nielsen et al. 2023). *A. salmonicida* is the primary causative agent of furunculosis in salmonids (Kaattari and Piganelli 1996). Acute furunculosis can involve septicemia with hemorrhages at the base of the fins, altered or reduced feeding behavior, and melanosis, while subacute furunculosis consists of lethargy, exophthalmia, and hemorrhaging in muscles and organs (Austin et al. 2007) (Søren Saxmose Nielsen et al. 2023). Mesophilic species such as *A. veronii* and *A. hydrophilia* were shown to cause hemorrhagic septicemia in freshwater crayfish (*Pacifastacus leniusculus*), carp (*Cyprinus carpio*), cod (*Gadus morhua*), and goldfish (*Carassius auratus*) (Søren Saxmose Nielsen et al. 2023). Goldfish furunculosis was first described in 1969 after lesions containing *A. salmonicida* were identified (Mawdesley-Thomas 1969;Igbinosa et al. 2017; Janda and Abbott 2010). Early cutaneous infections involved the appearance of white proliferation on the epithelium, followed by peripheral hemorrhage beneath scales, lesion development, loss of scales, necrosis of dermis and degeneration of underlying

muscle (Mawdesley-Thomas 1969). Septicemia occurs in larger and deeper legions if the infection is left untreated (Mawdesley-Thomas 1969; Adams and Adams 1996). Untreated infections cause histological changes such as decreased hemocyte aggregation in the digestive system, and increased hemocyte aggregation and necrotic cells in the gills of infected Catfish (Mulia et al. 2023). The damage of the cutaneous layer results in immune responses, marked by the infiltration of leukocytes to the site of infection, consisting primarily of macrophages and neutrophils, followed by lymphocytes. These leukocytes remain at higher than baseline levels until up to 21 days post infection (Mulia et al. 2023).

In response to *Aeromonas* infection, many teleost fish will increase pro-inflammatory cytokines for the recruitment and increased function of leukocytes (Zou and Secombes 2016). Key cytokines induced by bacterial infection include TNFα, IL-1β, and IL-6 (Zou and Secombes 2016; Soliman and Barreda 2023). The induction of pro-inflammatory cytokine production is followed by a phagocytic response. Pathogen clearance often occurs within the first week of a subcutaneous infection. Following pathogen clearance, the underlying damaged tissue layers are repaired (Guo and DiPietro 2020).

1.5.2 Zymosan

1.5.2.1 Recognition of Zymosan by Host Immune System

Zymosan is an insoluble β-1,3-glucan polysaccharide that contributes to the structure of the cell wall of yeast (*Saccharomyces cerevisiae*) and it is commonly used in immune-challenge experiments as a pathogen mimic, specifically, a fungal mimic (Ohno 2007). In mammals, zymosan detection primarily occurs via Toll-like receptor TLR2 and TLR6 heterodimers (Sato et al. 2003; Arya et al. 2023). TLR2 and 6, along with dectin-1, are receptors found on the surface of mammalian macrophage and neutrophils (Arya et al. 2023). Dectin-1 functions to phagocytose zymosan and other β-glucans, and the TLRs function to trigger an inflammatory response. Binding of zymosan by Dectin-1 is followed by SYK (spleen tyrosine kinases) recruitment and activation that facilitates phagocytosis and the respiratory burst response of macrophages and neutrophils (Underhill et al. 2005). Binding of zymosan to TLR2 and TLR6 induces intracellular signalling through the MyD88 pathway that promotes cytokine secretion for a subsequent pro-inflammatory response mediated by AP1 and NFκB (Sato et al. 2003; Colleselli et al. 2023).

1.5.2.2 Zymosan Induction of Acute Inflammatory Responses

In goldfish, several studies using intraperitoneal injection of zymosan have shown that fish had robust and quick responses when compared to mammals (Rieger et al. 2012). Specifically, fish exhibit an earlier recruitment of leukocytes, both myeloid and lymphoid populations. In carp and goldfish, zymosan injection induced oxidative burst and nitric oxide responses, and secretion of TNFα, IL-1β, IL-6 and IL-11 suggesting conserved responses to certain PAMPs (Pietretti et al. 2013; Rieger et al. 2012). Furthermore, it is now evident that carp amongst other teleost can detect β-glucan through TLR and non-TLR receptors, and β-glucan immune challenge results in increased ROS and NO production (Pietretti et al. 2013). Through similar pathways, immune challenge with zymosan in fish results in upregulation of key cytokines including: TNFα, IL-1B, and IFNγ along with an increase in phagocytic uptake and respiratory burst (Haddad et al. 2023).

1.5.3 Other Pyrogens Encountered by Fish

While zymosan is widely used in the induction of acute inflammation in many mammalian and non-mammalian vertebrates, there are other pathogens that a goldfish can encounter in their natural environments that are not explored within the scope of my project. Although *A. veronii* is a common freshwater pathogen encountered by fish in natural settings, there are other bacterial species that infect fish. The classes of pathogens that a fish may encounter in natural settings include fungi, bacteria, parasites, and viruses. The following subsections include general information about some pathogens that aforementioned fish populations may be exposed to and infected with.

1.5.3.1 Exogenous fungal species

Infection by fungal and fungal like pathogens in animals has been on the rise, and most of these species infect and cause disease in fish from multiple different families (Gozlan et al. 2014). For example, *Sphareothecum destruens* (a fungal like pathogen) infects *Oncorhynchus mykiss* and *Salmo salar* and causes up to 90% mortality in fish populations after 20-30 days of infection (Mendonca and Arkush 2004). Unfortunately, there are no visible external symptoms of this infection and currently the only method to confidently identify this pathogen is through PCR after mortality (Mendonca and Arkush 2004). With increased monitoring in aquaculture settings, there are new hosts for fungal and fungal-like pathogens being discovered daily(Gozlan et al. 2014; Fisher et al. 2020). For example, *Saprolegina ferax* infects *Carassius auratus* and

Oncorhynchus mykiss eggs, and *Saprolegina diclina* infects *Oncorhynchus mykiss*, and the eggs of *Salmo salar*. Many of these infections can be chronic and have no external symptoms and rather are identified after mortality of the fish. Fungal and fungal-like pathogens result in significant mortality of fish in both natural and aquaculture settings (Fisher et al. 2020).

1.5.3.2 Exogenous bacterial species

Many *Aeromonads* such as *Aeromonas hydrophila* and *Aermonas salmonicida* are known to cause furunculosis, septicemia and ulcerative diseases in fish populations including trout, salmon and goldfish (Irshath et al. 2023). As these bacteria thrive in both freshwater and saltwater, infections with this bacterium are frequently observed. Another prevalent bacterial pathology is vibriosis caused by bacterial species such as *Vibrio anguillarum, Vibrio salmonicida,* and *Vibrio carchariae* (Irshath et al. 2023). Vibriosis causes major decreases in aquaculture settings resulting in massive reductions in farmed fish such as salmon and rainbow trout. Interestingly, these massive die offs have promoted increased research around the world in fish vaccination against certain bacteria for the preservation of fish populations in aquaculture and farming settings.

1.5.3.3 Exogenous parasitic species

Parasitic species often alter the hosts' eating, mating, social behavior, and migration patterns to advance infection (Ezenwa 2004). Specifically in fish, some parasitic species have been reported to alter secretory functions, metabolism which are not easily observed, but external signs of parasitic infections can include damage of the skin and extensive furunculosis (Merck & Co 2024). Goldfish and koi carp (*Cyprinus carpio*) can be infected by parasitic fish lice (*Argulus japonicus*) (Wafer et al. 2015). These fish lice belong to a large group of crustaceans that cause diseases in fish. Additionally, parasitic fish lice also serve as a vehicle for other pathogens such as *Rhabdovirus carpio*, larval nematodes and the *Saprolegina* fungus. Symptoms of infection with fish lice include hemorrhaging, anemia, fin loss, lethargy, and loss of body integrity. Another common parasite in fish is the anisakis roundworm, which has been demonstrated to be enzootic and can impact humans if consumed (Aibinu et al. 2019). Anisakis roundworm species include *Anisakis simplex, Anisakis pegreffii* and *Anisakis physeteris*. Herring (*Clupea harengus*), cod (*Gadus morhua*), anchovy (*Engraulis encrasicolus*), Atlantic salmon (*Salmo salar*) and sea trout (*Salmo trutta*) are fish populations in which Anisakis roundworm is prevalent.

1.5.3.4 Exogenous virus infections

Fish can also contract viral infections often carried by parasites or from horizontal transfer from other infected fish. Viruses that can infect fish are classified in main categories including: rhabdoviruses, herpesviruses, reoviruses, retroviruses, iridoviruses, birnaviruses and orthomyxoviruses (Kim and Leong 1999). Infection with viruses can result in symptoms such as: necrosis of internal organs, tumors, and anemia. Viral hemorrhagic septicemia virus is a rhabdovirus that fish are often infected with. Susceptible species include Atlantic salmon, rainbow trout, goldfish, Chinook salmon and more. Viral hemorrhagic septicemia virus was first identified in Europe and results in significant loss of rainbow trout populations in fish farms (Faisal et al. 2012). Signs of infection include exophthalmia, pale gills, hemorrhaging at the fin base and internal organs and ultimately, mortality. In primary cell culture studies and cell line studies, this virus grows in fish cells optimally in temperatures between 10-15℃ and the virus loses infectivity at temoeratures above 20℃. Horizontal transmission of the virus occurs at temperatures between 1-12℃ and there is little transmission observed above 15℃.

1.6 CELLULAR COMPONENTS OF ACUTE INFLAMMATION

1.6.1 Innate Immune cells

In mammalian systems, upon tissue insult, macrophages are the primary professional phagocytes that arrive first at the site of insult and partake in phagocytosis of the pathogen, along with releasing pro-inflammatory cytokines. In goldfish (*Carassius auratus*), neutrophils are the most abundant phagocyte in response to tissue insult. Phagocytes are key to the detection and clearance of pathogens, activation of other immune cells, induction and resolution of inflammation, initiation and execution of tissue repair, and maintenance of tissue homeostasis in an organism (Chen et al. 2018). Even in non- mammalian vertebrate systems (trout, zebrafish, goldfish, and catfish) professional phagocytes are responsible the bulk of pathogen clearance (Uribe et al. 2011; Kordon et al. 2018). While many immune cells contain the basic cellular machinery to phagocytose, professional phagocytes have the capacity to remove the pathogens and present relevant antigens to lymphocytes with significantly greater speed and efficiency. To detect pathogens, the cells of the innate immune system recognize a variety of surface moieties on viruses, bacteria, and fungi by germline-encoded receptors on these cells called pattern recognition receptors (PRRs) (Thompson et al. 2011). These receptors can recognize conserved

domains of the pathogen moieties called pathogen associated molecular patterns (PAMPs). Common PAMPs include cell wall components of microbes, sugars and flagellin. Leukocytes such as macrophages, neutrophils, and monocytes ubiquitously express PRRs for detection of PAMPs, the binding of which initiates leukocytic anti-microbial responses. These antimicrobial responses include respiratory burst, release of reactive oxygen species (ROS), release of nitric oxide species (NO), fusion of the phagolysosome, neutrophil degranulation, and neutrophil extracellular traps (NETs) (Gierlikowska et al. 2021; Havixbeck et al. 2015). The antimicrobial response in acute inflammation is executed by a significant influx of leukocytes, a response that is primarily regulated through the release of cytokines that recruit and activate more leukocytes executed by the earliest responding leukocytes. Overall, these findings demonstrate the dynamic role of leukocytes and other cells in inflammation and the ever-evolving diverse roles of these cells (Rieger et al. 2012)

1.6.1.1 Neutrophils

Neutrophils are a leukocyte subset that are polymorphonuclear granulocytes that execute anti-microbial functions such as phagocytosis, pathogen capture, and release of neutrophil extracellular traps (NETs). Neutrophils are recruited by CXCL8, platelet activating factor (PAF), and lipid mediator B4 (LTB_4) (Sadik et al. 2011). In vertebrate studies, leukocyte populations are often distinguished by cell surface ligands. In mammals, neutrophil identification is oachieved using flow cytometric gating for cells positive for CD16 (FCγRIII) and CD62L (L-selectin) (van Staveren et al. 2018). However, due to the lack of annotated neutrophil specific markers in the goldfish genome, early characterizations involved separation of neutrophils from other hematopoietic subpopulations. As neutrophils are denser than macrophages, monocytes and lymphocytes, gravimetric centrifugation was utilized to separate "neutrophil like" cells. These cell fractions allowed for the development of flow cytometric parameters of neutrophil size and internal complexity (Katzenback and Belosevic 2009). Furthermore, authors established that goldfish neutrophils were capable of degranulation in response to pathogen detection, a critical step of NETosis in neutrophils for pathogen capture. Like mammalian neutrophils, goldfish neutrophils were capable of potent respiratory burst responses. Due to their granular enzymatic activities, goldfish neutrophils stained positive for Sudan Black-B and myeloperoxidase cytochemical stains (Katzenback and Belosevic 2009). In mammals, neutrophils are recruited from peripheral blood, whereas in goldfish they are recruited from circulation and the kidney, the

hematopoietic organ of fish (Katzenback et al. 2012; Havixbeck et al. 2015). Since these foundational characterizations, many other aspects of neutrophil functions and kinetics have been established in goldfish. Interestingly, unlike what is observed in mammals, teleost neutrophils make up less than 5% of circulating leukocytes at homeostasis (Havixbeck et al. 2015). The study of whole organism response to zymosan (fungal mimic) has allowed for the measurement of cell migration into the site of immune challenge. The peak of neutrophil infiltration has been identified at 16 hours post zymosan challenge in goldfish (Haddad et al. 2023). Once the neutrophils arrive at the site of challenge, they exert robust intracellular and extracellular antimicrobial responses required for pathogen clearance (Havixbeck et al. 2015). These responses include release of ROS/NO, NETs, and degranulation of granules containing cytotoxic enzymes such as myeloperoxidase, metalloproteinase, acid hydrolases, elastase, and proteinase-3 (Chen et al. 2021; Morel et al. 1991). Another key activity of neutrophils is phagocytosis, resulting in increased O_2 consumptions and eventual respiratory burst (Gierlikowska et al. 2021). Interestingly, in addition to promoting inflammation, goldfish neutrophils have been shown to play a critical role in the resolution of acute inflammation. This is accomplished via a transition from LTB_4 production to LXA_4 production (Havixbeck et al. 2015). Due to the high presence and activity of neutrophil granules, we can identify neutrophils using staining techniques such as the SUDAN Black-B stain (Blumenreich 2011). This stain adheres to acidic components, and therefore make a neutrophil appear a cloudy grey color due to the staining of acidic granules.

1.6.1.2 Macrophages and Monocytes

In mammals, monocytes are classically known to be a circulating leukocyte subset that can differentiate further into macrophages and dendritic cells. These migrating cells can preform specific immune functions such as phagocytosis, ROS release, immune cell recruitment and promotion of tissue repair (Parihar et al. 2010; Lu and Chen 2019)). Chemokines such as CCL2 and CCL7 are key in monocyte recruitment during inflammation (Shi and Pamer 2011). While most cells can respond to an inflammatory site and release CCL2, neutrophils hold the greatest contribution in recruiting monocytes (Shi and Pamer 2011). To differentiate mammalian monocytes from other leukocyte cell subpopulations, flow cytometry gating for cell-surface antigens is utilized. Classical monocytes are often identified by a high expression of CD14, and no expression of CD16 (CD14+/CD16-) (Italiani and Boraschi 2014). Macrophages are a specialized leukocyte subset that is involved in the detection, capture, and clearance of

pathogens. In addition to being an important effector cell within the innate immune system, mammalian macrophages are antigen presenting cells, often communicating with the cells of the adaptive immune system. CD14 is a surface lipopolysaccharide-binding protein highly expressed on monocytes and tissue resident macrophages (Naeim et al. 2018) (Cutler and Davies 1998). CD16 is type I transmembrane receptor containing 2 Ig -like domains which are involved in mediating an antibody-dependent cell-mediated cytotoxicity (Naeim et al. 2018).

The classically known function of macrophages was phagocytosis and release of inflammatory cytokines, however, the current paradigm indicates that macrophages can be differentiated and polarized from monocytes to various phenotypes by a variety of stimulants often resulting in either pro-inflammatory or pro-resolving phenotypes (Watanabe et al. 2019; Austermann et al. 2022) (Rieger et al. 2014). This determines whether the macrophage will execute its antimicrobial function and release cytokines such as TNFα, IL1β, IL-6 or execute tissue repair and pro-resolution functions and release cytokines such as TGFβ and IL-10 (Arango Duque and Descoteaux 2014). Macrophages in mammalian studies are often identified via flow cytometry by high expression of CD115, the macrophage colony stimulating factor receptor (MCSF-R), macrophage mannose receptors (MMR) and CD14 present in tissue resident macrophages. However, unlike mammals, in teleost, CD14 is not in the genome making it increasingly difficult to identify and distinguish macrophage and monocyte populations (Novoa et al. 2009). Many mammalian cell surface markers are not identified or annotated on the goldfish genome, posing a significant challenge in distinguishing leukocyte subpopulations. Early studies in the field aiming to characterize goldfish macrophages used macrophage growth factors (MGF) from a goldfish macrophage cell line to stimulate cells in the hematopoietic organ (kidney) to differentiate into primary kidney macrophages (PKM) (Neumann et al. 1998) (Neumann et al. 2000). Despite the lack of antibodies binding to proteins on the surface of PKMs, authors were able to distinguish distinct macrophage populations via flow cytometry by gating for size and internal complexity. Furthermore, the greatest proportion of PKMs were found in two clusters termed region (R)2 and R3. R2 and R3 PKMs were stimulated with macrophage activating factors (MAF) and LPS and were able to produce a robust antimicrobial respiratory burst response. After cytochemical staining of these populations, authors deduced that the R2 subpopulation was capable of potent non-specific-esterase activity and potent NO responses after stimulation, which differs from mammalian macrophages that lose non-specific

esterase activity. Morphologically these R2 cells appeared similar to tissue macrophages with irregular shapes, extensive vacuolization and a substantial cytoplasm. The R3 subpopulation morphologically and cytochemically appeared as monocytes, and functionally had a comparatively weaker respiratory burst response than the R2 subpopulations. The separation of monocytes from macrophages allowed for the isolation of macrophages and the identification of a receptor found on macrophages and monocytes in fish, the colony stimulating factor receptor 1 (CSF-1R) and a soluble form of CSF-1R expressed by early progenitor cells and macrophages in PKM cultures to modulate proliferation and self-renewal (Barreda et al. 2005; Hanington et al. 2007). While many others have tried to identify the two cell subpopulations using surface markers, the simplest technique remains histological staining and enumeration. Due to macrophages and monocytes lack of granular enzymes, they appear clear on Sudan Black B stain (Blumenreich 2011). In the Wright-Geimsa stain, macrophages are smaller and contain smaller nuclei than monocytes (Cornbleet 1998; Haddad et al. 2023). Post immune challenge with zymosan, macrophages and monocytes also peak at 16 hours, but do not make up as great of a portion of the infiltrating leukocytes as neutrophils (Haddad et al. 2023).

1.6.1.3 Lymphocytes

While innate immune responses are critical in pathogen clearance, communication with the adaptive immune system allows faster host responses in subsequent attacks by the same pathogen. Lymphocytes are integral to the adaptive immune response and contain subpopulations such as B cells, NK cells and T cells (Alberts et al. 2015). While T cells are primarily responsible for cell mediated immunity, B cells contribute to humoral immunity via their production of antibody proteins (Elena and Lopera 2013). Higher vertebrates, such as humans have B cells with the capacity of robust memory responses and highly specialized B and T cell subsets, while lower vertebrates have a more rudimentary adaptive immune system (Flajnik 2018). For example, germinal centres (structures where B cells can generate high-affinity antibodies) are believed to have been present in evolution for the last 250 million years, and have not been identified in reptiles, amphibians, or fish (Klein and Dalla-Favera 2008; Flajnik 2018). Furthermore, class switch recombination (CSR) allows a B cell to generate immunoglobulin of another class while retaining the same affinity to an antigen, allowing for interaction with different effector cells. CSR systems are not present in fish and first appeared in amphibians around 350 million years ago (Flajnik 2018. Lymphocytes in vertebrates and mammals are also

characterized using surface receptors for high-throughput analysis such as CD35 (C3b/C4b receptor), CD30 (tumor necrosis factor receptor involved in TCR-mediated cell death), CD223 (lymphocyte activation gene 3) are a few examples. In teleost, cytotoxic cells such as NK cells and T cells have been identified in several species in the 1990's in carp, rainbow trout, zebrafish, and catfish. The creation of multiple leukocyte cell lines from channel catfish (*Ictalurus punctatus*) and crucian carp (*Carassius auratus langsdorfii*) allowed for the identification of lymphoid cell populations in teleost (Hogan et al. 1996;Hasegawa et al. 1998). Due to lack of specific antibodies targeting lymphocytes, the next best reliable method for detection of lymphocytes was using Wright-Geimsa staining in which they appear to have very little cytoplasm and are small in size relative to macrophages and neutrophils (Witeska et al. 2022). After immune challenge with zymosan, lymphocytes steadily increase 20 hours post injection and remain relatively high throughout the 48-hour observation window (Haddad et al. 2023.

1.6.1.4 Platelets and Thrombocytes

One of the first reports of platelets was done by Giulio Bizzozero in 1882 where he described small translucent spherules that formed granular masses when they passed by small arterial punctures (Ribatti and Crivellato 2007). After carefully studying platelet function microscopically, he determined the role of platelets in coagulation, aggregation, and subsequent fibrin formation (Ribatti and Crivellato 2007). Since then, countless studies have contributed to the understanding of the classical function of platelets: hemostasis. Hemostasis is delineated into distinct cellular and molecular events involving tethering to the site of injury, activation and recruitment of other platelets, aggregation and subsequent fibrin formation (Tomaiuolo et al. 2017). Platelet activation occurs through multiple triggers, one of which includes the interaction of platelet receptors GP_{1b}/V/IX to subendothelial extracellular matrix (ECM) components binding to von Willebrand Factor (vWF) (Nieswandt et al. 2011; (Lam et al. 2015)). Another trigger is GPVI and $\alpha_{IIb}\beta_3$ binding to collagen in the ECM (Bergmeier and Hynes 2012). After the initial tethering of platelets to the site of injury, there is a morphological change in platelets going from a round shape to a flattened shape (Rumbaut and Thiagarajan 2010 Tomaiuolo et al. 2017). This flattened shape allows for a stronger adhesion after which a greater population of platelet is recruited through the integrin $\alpha_{IIb}\beta_3$ receptor (Sorrentino et al. 2016).

Prior to the 1950's, platelets were known to be non-nucleated cells that were fragments of megakaryocytes, but the metabolic or cellular triggers to increase production of platelets was unknown. In 1958, in a simple experiment where the blood of rats with thrombocytopenia (low thrombocyte count) was transfused into healthy control rats, resulting in these control rats producing more platelets hinted at a regulator of thrombopoiesis (formation of thrombocytes) (Kelemen et al. 1958). However, a purified substance was not produced despite strenuous efforts. While scientists were still working to identify the regulating cytokine or protein for thrombopoiesis, other platelet functions were coming to light (Kaushansky 2005). It wasn't until 1990, in a completely different field of study of a myeloproliferative leukemia virus, where abnormalities in an oncogene *v-mpl* and a cellular *c-mpl* were observed (Michèle Souyri et al. 1990; Vigon et al. 1992). In subsequent years, this *c-mpl* receptor was identified to have a key role in maintaining thrombocyte populations and its corresponding ligand, thrombopoietin was subsequently characterized (Kaushansky 2005).

The identification and characterization of thrombocytes in teleost fish was initially reported in zebrafish (Jagadeeswaran et al. 1999). The authors concluded that the zebrafish thrombocyte was a hemostatic homologue of the mammalian platelet in addition to morphological and functional similarities (Jagadeeswaran et al. 1999). Since then, thrombocytes have been identified in other teleost species such as carp, rainbow trout, goldfish, catfish, and many others (Stosik et al. 2019; Grant 2015; Tavares-Dias and Ruas 2007; Katzenback et al. 2016). Concurrent with identification of fish thrombocytes, there has been an expansion of our understanding of avian and amphibian thrombocytes, which also show similar conserved morphology to teleost thrombocytes (Ferdous and Scott 2023; Ferdous 2014). While there are some functional similarities between mammalian platelets and fish thrombocytes, some key differences exist. Thrombopoiesis processes, cell morphology, activation stimuli, interactions with immune cells and effects of antipyretics of thrombocytes and platelets show some degree of conservation highlighted in **Table 1.6.**

Table 1.6 Functional and phenotypic attributes of mammalian platelets and teleost thrombocytes

Function or phenotype	Mammalian (Human)	Fish
Progenitors	• Fragments of megakaryocytes	• Myeloid early progenitors (Katzenback et al. 2016)
Site of Development	• Megakaryocytes (Levin 2019) • Site of differentiation: bone marrow (Levin 2019)	• Hematopoietic compartment: kidney (Katzenback et al. 2016)
Organelles	• Platelet-specific granules: α-granules, lysosomal granules, dense granules (Lam et al. 2015) • Non-nucleated • Contain main organelles of megakaryocytes. • Change shape when activated and engaged in clot (Rumbaut and Thiagarajan 2010; Tomaiuolo et al. 2017)	• Nucleus: oval or spindle shaped, can change shape when activated (Claver and Quaglia 2009) • Carbohydrate-rich cytoplasm • Acidophilic granules in the cytoplasm (Watson et al. 1963) • Lysosomal granules with lysozyme and inducible iNOS (Nagasawa et al. 2014) • Intracellular vacuoles (Stosik et al. 2019)
Identification methods	• PAS stain (Pogorelov et al. 2011) • Giemsa Stain (Prasad et al. 2012) • Leishman's staining (Umashankar et al. 2014) • Flow Cytometry: P-selectin, $\alpha_{IIb}\beta_3$ receptor, GP_{1b}/V/IX receptors (Bergmeier and Hynes 2012; Sorrentino et al. 2016)	• MPO stain (Ksenija Aksentijević et al. 2023) • PAS stain (Ksenija Aksentijević et al. 2023) • Giemsa Stain • Test for acid phosphatase (ACP) (Wang et al. 2021) • Test of a-naphthyl acetate esterase (ANEA) (Wang et al. 2021) • Flow Cytometry: Dil+, GB10 mAb (Nagasawa et al. 2014)

	• Functional responses such as adherence, recruitment and apoptosis measured through sandwich ELISA, aggregation assays, and transcriptomics (Gowert et al. 2014; Burkhart et al. 2012))	• C-mpl and CD41 ligand gene expression (Stosik et al. 2019) • Activation: *tpo* upregulation in different tissues in response to stimuli
Recruitment/Proliferation events	• Injury and damage to vasculature cells • Activated platelets recruit other activated platelets through integrin $\alpha_{IIb}\beta_3$ receptor (Bergmeier and Hynes 2012; Sorrentino et al. 2016)	• GpIIb/IIIa and GpIb receptors responding to agonists such as ADP and collagen (Thattaliyath et al. 2005) • Loss of blood causing *gata*1 and *epo* upregulation (Jagadeeswaran et al. 2010; Katakura et al. 2015)
Activation events	• Soluble agonists: ADP, TXA_2, Thrombin (Tomaiuolo et al. 2017) • Exposed collagen in ECM binding to GP_{1b}/V/IX and $\alpha_{IIb}\beta_3$ receptors (Bergmeier and Hynes 2012; Sorrentino et al. 2016)	• Release of Ca^{2+} in response to ADP agonist (Thattaliyath et al. 2005) • Annexin-V binding (apoptosis) (Khandekar et al. 2012) • P-selectin binding to recruit other cells and roll between endothelium cells (Thattaliyath et al. 2005) • Release of microparticles (micro-vessicles) for recruitment and further activation (Khandekar et al. 2012)
Classical Functional Responses	• Hemostasis • Aggregation and multi-layer thrombus formation (Tomaiuolo et al. 2017) • Promotion of wound repair	• Hemostasis via *gata*1, *lmo*2, *epo* • Multi-layered thrombus formation (Tavares-Dias and Ragonha 2009)

Non-classical functions	• Autophagy (Banerjee et al. 2019) • Release microparticles and microRNAs that allow to alter function of other cells in the(Lam et al. 2015)	• Phagocytosis (Nagasawa et al. 2014) • Apoptosis to release microparticles for further thrombocyte activation (Khandekar et al. 2012)
Innate Interactions	• Interaction with neutrophils and monocytes once activated using P-selectin and PSGL-1 cross linking(Zarbock et al. 2007). • Increase GpIba interaction with PSGL-1 and LFA1 to increase neutrophil NETosis(Kral et al. 2016) • Release CXCL4 from α-granules to recruit neutrophils(Lievens et al. 2014). • CXCL1, PF4, CXCL5 CLCL7 and IL8 and CXCL12, and MIP1a and RANTES(Lam et al. 2015)	• IL-1β, IL-6, TNF-α transcripts, role in pro-inflammation (Stosik et al. 2019) • CXCL8 and CXCAL1 release of chemokines to recruit neutrophils. (Stosik et al. 2019) • IL-10, SOCS3-1,2, SOCS3-3,4 genes, role in anti-inflammation (Stosik et al. 2019)
Adaptive Immune interactions	• Communicate with lymphocytes once activated (Pitchford et al. 2003). • Release CD40L acting on B and T cells (Lam et al. 2015). • Release PF4 and RANTES to increase interaction and activation of T cells	• MHCII –the mRNA for this can increase which means they can function as APCs that present antigen to T lymphocytes (Stosik et al. 2019). • Many TLRs for recognition of PAMPs and specific responses to antigens (Stosik et al. 2019)
Interaction with antipyretics	• Ibuprofen inhibits thromboxane levels and	• Unknown

	platelet functions(Kaehler et al. 2007) • Multiple NSAIDs result in decreased thromboxane levels resulting in increased hemorrhage in surgery(Raineri-Gerber and von Felten 1991) • Ibuprofen causing life threatening thrombocytopenia in rare cases(Sanjay Kumar Jain 1994)	

1.7 Soluble Factors in Acute Inflammation

1.7.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are defined as semi-reduced metabolites of oxygen with a potent capacity to oxidize cellular lipid components, proteins, and DNA (Ryter et al. 2007). At high concentrations, ROS can be very damaging leading to oxidation of cellular macromolecules and subsequent loss of cell viability, whereas at low concentrations they can act as signalling molecules in complex pathways with functions such as protein phosphorylation, and regulation of growth factors and transcription factors (Cai et al. 2003). ROS are generated endogenously in response to various stimuli such as cytokines and growth factors. The two main sources of ROS production are through cytochrome P450 in the ETC of mitochondria, and NADPH and Dual oxidases (Nox)(Duox), which are present in professional phagocytes and endothelial cells (Pendyala and Natarajan 2010). The increased and sustained production of ROS has been linked to chronic inflammatory pathophysiology, indicating the importance of a proper resolution of the ROS response in returning to homeostasis (Lambeth et al. 2007).

1.7.2 Nitric Oxide Species (NO)

In immunity, Nitric oxide (NO) is generated by the inducible nitric oxide synthase enzyme (iNOS). The release of NO by immune cells can induce damage to critical cellular components of both host and pathogen cells, namely fragmentation of DNA and deamination of

nucleotides in proteins (Weitzman and Weitzman 2014). Therefore, NO release is an antimicrobial response mainly used in the inflammatory process by neutrophils and macrophages.

In addition to its role in inflammation, NO plays various roles in tissue repair, particularly in the proliferation and tissue remodeling phases (Luo and Chen 2005). NO released through the iNOS enzyme regulates collagen deposition, cell proliferation and wound contraction within the tissue repair process (Witte and Barbul 2002).

1.7.3 Cytokines and Chemokines in Acute Inflammation

While many cytokines are involved in acute inflammation and throughout all phases of tissue repair, I selected TNFα, TGFβ, IL-1β and IL-10 as representative pro- and anti-inflammatory cytokines to examine throughout the study.

1.7.3.1 Pro-Inflammatory Cytokines

1.7.3.1.1 TNFα

Tumor necrosis factor alpha is soluble protein first identified in 1975, demonstrating at certain concentrations to cause regression of tumors while having cytotoxic effects at higher concentrations (Cechin and Buchwald 2014). Later, in the 1990's, another structural and functional related protein, lymphotoxin, was further characterized and coined TNFβ. These pear-shaped trimers bind to TNFR-1 and TNFR-2 receptors to execute an inflammatory response in immunity (Parameswaran and Patial 2010). In mammals, TNFα is predominantly produced by macrophages, however other cells such as NK cells, fibroblasts, cytotoxic T cells and B cells have the ability to release smaller quantities of this proinflammatory protein. Functionally, TNFα triggers the release of inflammatory cytokines and chemokines that affect the function, proliferation, and recruitment of other leukocyte subsets, making it an ideal representative pro-inflammatory cytokine. The binding of TNFα to the mammalian predominant TNFR-1 results in TRADD binding and the formation of various complexes (I, IIa, IIb, IIc). Downstream of complex I signalling pathway is induction of inflammation, tissue degeneration, cell proliferation and survival, and host defence responses. Complex IIb and IIc result in activation of caspase 8, a critical component of the apoptotic pathway (Jang et al. 2021). Due to roles in both promotion of inflammation and tissue

degeneration, chronic upregulation of TNFα often results in chronic inflammatory pathophysiology in humans such as rheumatoid arthritis and inflammatory bowel disease (Chen et al. 2018). TNFR-1 receptors are expressed on the surface of most cells within the body and therefore TNFα has varying functions, rendering it a potent pleiotropic inflammatory mediator (Gough and Myles 2020).

In teleost fish, the first identification of TNFα was reported in Oliver flounder (Ikuo Hirono et al. 2000). Since then, many other members of the TNF superfamily have been characterized in multiple teleost species (Hong et al. 2013). Numerous isoforms of the TNFα genes have been identified in species such as common carp, rainbow trout and blue fin tuna (Saeij et al. 2003; Laing et al. 2001). In goldfish, two isoforms of TNFα have been identified at the gene and protein levels, that are phylogenetically most homologous to carp TNFα-1 (Grayfer et al. 2008). When macrophages are activated, a significant upregulation of both isoforms is observed (Grayfer et al. 2008). The recombinant TNFα2 molecule instigates dose dependent responses in primary goldfish macrophages. Recombinant-TNFα-2 has been shown to prime respiratory burst of monocytes and induce NO production in primary macrophages. TNFα in goldfish is a central effector cytokine that regulates inflammatory and antimicrobial responses. Due to the potent capacity of TNFα to not only regulate leukocyte recruitment but also effector functions at multiple levels of inflammatory signalling pathways, TNFα is an ideal candidate for determining pro-inflammatory upregulation in the site of immune challenge (ie. furuncle).

1.7.3.1.2 IL1β

Interleukin 1β is a potent mediator of inflammatory responses and host resistance to pathogens. It is involved in essential tissue repair processes and can exacerbate damage during tissue injury in both acute and chronic wounds (Lopez-Castejon and Brough 2011). Multiple mammalian cells produce and release IL1β, particularly circulating sensors that detect PAMPs. In mammalian systems, mature IL1β is produced when pro- IL1β is cleaved by Caspase-1, which is activated by inflammasome formation (Franchi et al. 2009). IL1β is also secreted via microvesicles, and lysosome exocytosis during pyroptosis (a significantly faster variation of apoptotic cell death often activated in response to DAMPs and PAMPs) (Piccioli and Rubartelli 2013). Many have suggested that the mechanisms of IL1β release can be best understood as a

continuum of release depending on the strength and duration of the inflammatory stimulus, type of effector cell, and the tissue microenvironment (Lopez-Castejon and Brough 2011; Piccioli and Rubartelli 2013). Due to the predominance of IL1β receptors, macrophages and monocytes are most sensitive to IL1β release.

In goldfish IL1β was the first characterized cytokine from the interleukin family (Grayfer et al. 2018). Recently two isoforms of IL1β have been classified into IL1β type 1 and IL1β type 2 (Zou and Secombes 2016). Like mammalian systems, both Caspase 1 and Caspase 8 can sleave pro-IL1β to mature IL1β (Angosto et al. 2012). Responding cells have a heterodimeric receptor protein that binds to IL1β resulting in activation of pathways for phagocyte recruitment and activation of lysozyme mobilization in activated phagocytes (Zou and Secombes 2016). While IL1β release directly allows for recruitment and antimicrobial mechanism activation in macrophages, IL1β release can also augment chemokine production in cells at the infection site, such as CXCL8 recruitment of neutrophils (Peddie et al. 2001). Furthermore, this potent cytokine has been shown to regulate the inflammatory process by consistently antagonizing potent anti-inflammatory molecules such as TGF-β (Wang et al. 2016). Therefore, IL1β release impacts essential processes such as the resolution of inflammation along with the transitions through the different phases of tissue repair, making it an ideal candidate for the measurement of inflammatory agendas and progression through phases of tissue repair in sites of immune challenge (Soliman et al. 2021).

1.7.3.2 Anti-inflammatory mediators

1.7.3.2.1 TGF-β

Members of the transforming growth factor superfamily can display various roles within immunity and overall homeostasis within the body. Transforming growth factor-beta (TGF-β) is a pleiotropic anti-inflammatory polypeptide growth factor released and sensed by many leukocytes making it an ideal marker of pro-resolution in sites of immune challenge (Zou and Secombes 2016). The TGF-β molecule binds to T2R receptors resulting in the phosphorylation and subsequent activation of the T1R receptor (Wrana et al. 1994). The activation of both receptors results in signalling cascades and downstream activation of regulator proteins integral to cell differentiation, and the chemotaxis and activation of leukocytes involved in the resolution of

inflammation (Tzavlaki and Moustakas 2020). TGF-β in mammals can have an overall anti-inflammatory effect by inhibiting cytotoxic cell activation. TGF-β primarily inhibits M1-like macrophages while activating M2-like macrophages (Gong et al. 2012). Within essential processes such as tissue repair, TGF-β promotes angiogenesis to support proliferating cells (Soliman et al. 2021).

TGF-β is highly conserved in avian, fish and mammalian organisms with avian and fish species exhibiting similar patterns of production, release, and effects on immune cells (Zheng et al. 2018). In fish, recombinant TGF-β was shown to inhibit the antimicrobial NO response of activated macrophages and in carp, TGF-β antagonized pro-inflammation via inhibition of TNFα and IL1β (Wang et al. 2016). In processes such as tissue repair, TGF-β increased cell viability of peripheral blood leukocytes and differentiation of fibroblasts, concurrent with suppression of erythropoiesis (Yang et al. 2012). Increased fibroblast proliferation has been demonstrated to result in increased collagen production in multiple models including fish, thereby exhibiting a conserved function in tissue repair (Haddad et al. 2008). Thus assessment of TGF-β allows for not only measurement of pro-resolution agendas within sites of immune challenge but can be an indicator of successful tissue repair.

1.7.3.2.2 IL-10

Interleukin 10 (IL-10) belongs to the type 2 α-helical cytokine denomination. IL-10 acts as an inflammatory suppressor in mammals and fish and is regarded as an anti-inflammatory cytokine (Voßhenrich and Di Santo 2002; Grayfer et al. 2011). IL-10 is produced by multiple cell types including T-cells, B-cells, epithelial cells, monocytes/macrophages, and NK cells in mammals. Following IL-10 release, this protein binds to the IL-10 receptor complex consisting of the IL10R1 and IL10R2 subunits (Shouval et al. 2014). The primary targets of IL-10 production are of macrophage/monocyte lineage while an indirect byproduct is the inhibition of lymphocyte function (Shouval et al. 2014). The inhibition of macrophage/monocyte cells results in an overall downregulation of ROS production and NO intermediate production, achieved by inhibition of TNFα synthesis.

In fish, IL10 has been shown to supress immune responses, inhibit inflammation, and antagonize multiple pro-inflammatory chemokines and cytokines. While IL-10 has been identified in multiple fish species, the functional consequence of IL-10 release have not been examined in

all species. Expression of IL-10 mRNA in goldfish is highest in the spleen, peripheral blood leukocytes and granulocytes (Grayfer et al. 2011). The addition of recombinant IL-10 reduces the expression of pro-inflammatory mediators such as TNFα, IL-1β and CXCL8 (Grayfer et al. 2011). Some of the central mechanisms of IL-10 in mammals is the binding of SOCS3 to JAK kinase thereby inhibiting STAT3 production and subsequent inhibition of cell differentiation (Carow and Rottenberg 2014). The potent anti-inflammatory effects and antagonism of pro-inflammatory cytokines accomplished by IL-10 makes it an ideal candidate for assessing activation and maintenance of anti-inflammatory resolution pathways throughout the tissue repair timeline.

1.8 THE ROLE OF ACUTE INFLAMMATION IN TISSUE REPAIR

1.8.1 The tissue repair process

Tissue repair is a fundamental process amongst multicellular organisms and consists of highly programmed phases (Gurtner et al. 2008). These phases are hemostasis, inflammation, proliferation, and tissue remodeling (Guo and DiPietro 2020). For precise tissue repair, all four phases must occur in sequence and within a specific time frame. Each phase of tissue repair involves the recruitment and function of various cell types, proinflammatory and anti-inflammatory cytokines, along with the production and deposition of important biomolecules (Guo and DiPietro 2020). The site of injury itself is host to a dynamic array of cells, cytokines, and biomolecules, balanced and regulated by host driven tissue repair mechanisms. As multiple stages are involved in tissue repair, the dysregulation of a single phase can result in improper wound closure, and chronic tissue pathology.

1.8.2 Stages of Tissue Repair

1.8.2.1 Hemostasis

Immediately following an injury, the first step of tissue repair is hemostasis. To prevent excessive blood loss and further pathogen entry, the host body generates a hemostatic plug. Hemostasis or cessation of blood loss in mammals is primarily accomplished by platelets after the detection of subendothelial elements of the host. Upon activation, platelets change shape, degranulate, release proteins and growth factors, and cytokines for the recruitment of other innate

immune cells alongside initiating thrombogenesis and coagulation pathways to reduce blood loss (Periayah et al. 2017). With detection of PAMPs and/or DAMPs, leukocytes are rapidly recruited to the site of injury (Gurtner et al. 2008). The presence of a clot and recruitment of immune cells by cytokines originating from platelets allows for the transition from the hemostatic phase to the inflammatory phase in tissue repair (Gonzalez et al. 2016).

1.8.2.2 Inflammation

Concurrently with the recruitment of leukocytes by both platelets and PAMP detecting cells is the induction of acute inflammation (Gurtner et al. 2008). This involves the recruitment of phagocytes for pathogen clearance accomplished by an array of pro-inflammatory mediators (Grose and Werner 2004). Succeeding pathogen clearance, multiple pathways are employed to regulate the resolution of inflammation. The tight regulation of acute inflammation, and timed shift to resolving inflammation is required to avoid defective tissue repair, and chronic inflammatory pathologies (Soliman et al. 2021). An increase in bodily temperature is observed in this phase of tissue repair and temperature has previously been demonstrated to impact the outcomes and efficiency of the acute inflammatory response (Haddad et al. 2023). Once the resolution of inflammation is nearly complete, the next phase of tissue repair begins.

1.8.2.3 Proliferation

The proliferation phase of tissue repair overlaps with the end of the inflammatory phase and this is primarily due to the pleiotropic activities of certain pro-resolving cytokines that also play a role in promotion of cell proliferation (Landén et al. 2016). To compensate for the loss of host cells in pathogen clearance in the inflammatory phase, the proliferation phase involves the regeneration of the hematopoietic pool. Furthermore, the proliferative phase stimulates the proliferation of epithelial cells to repair the epithelial layers damaged in the course of the infection (Schultz et al. 2011). Proliferation is accomplished by key cytokines such as vascular endothelial growth factor (VEGF) and endothelial growth factor (EGF) (Landén et al. 2016). Further, other anti-inflammatory cytokines such as TGF-β and IL-10 are upregulated for promotion of proliferation and to limit the upregulation of pro-inflammatory cytokines (Landén et al. 2016). After repairing the epithelial layer, the tissue remodelling phase begins.

1.8.2.4 Tissue remodelling

In contrast to the proliferation phase of tissue repair that involves robust proliferation and synthesis of extracellular matrix proteins which can take roughly 4-7 days on minor injuries in humans, full tissue remodelling can take months (Schultz et al. 2011). The aim of tissue remodelling is to repair the layers and architecture of the tissue with synthesized ECM such that it can be comparable to pre-injury conditions (Krafts 2010). A key molecule involved in tissue remodelling is collagen.

1.8.2.4.1 Collagen

Collagens are a group of proteins found throughout the body and in the context of a healing wound, collagen is produced by fibroblasts (Mathew-Steiner et al. 2021). Throughout tissue repair, collagen is deposited in and around the wound, however, for complete tissue remodelling, multiple rounds of collagen turnover are required (Mathew-Steiner et al. 2021). The initial deposition of collagen occurs in the proliferation phase by fibroblasts. These immature collagen molecules slowly begin to form covalent cross-links which allows for maturation of collagen into complex structures that can contribute to the strength and architecture of the tissue (Xue and Jackson 2015). Immature collagen examined under the microscope in tissue cross sections stained with Masson's Trichrome can appear as a thin layer, while mature collagen appears as stacked layers (Fang et al. 2019). Multiple factors can impact collagen deposition and maturation such as fibroblast inactivation due to sustained inflammation and cytokine interference of collagen maturation (Guo and DiPietro 2020).

1.8.3 Cells involved in tissue repair

There are numerous cell subpopulations involved in tissue repair. Within mammalian tissue repair models, tissue repair involves innate immune cell pathogen clearance along with adaptive immune cell recruitment to allow for earlier recognition in subsequent infections. In fish, our lab and others have identified the critical role of macrophages/monocytes and neutrophils in pathogen clearance against both fungal mimics and bacterial infections.

1.8.3.1 Fibroblasts

Fibroblasts are diverse mesenchymal cells with versatile roles throughout the host body mainly, contributing to connective tissue formation (Plikus et al. 2021). Activated fibroblasts in humans have been shown to contribute to ECM homeostasis, generate mechanical force within muscles, regulate tissue metabolism, participate in tissue regeneration, and play an immunomodulatory role with the cells in the innate immune system (Castillo-Briceño et al. 2011). While there are no current studies that have isolated primary fibroblasts in teleost, numerous investigations on fish fibroblast cell lines have led to the identification of fibroblast growth factors, collagen motifs in tissue repair, and fibroblast interaction with innate immune cells (Castillo-Briceño et al. 2011).

1.8.3.2 Platelets

Platelets are the primary instigators of the hemostatic plug and one of the first chemoattractant to recruit innate immune cells to the site of injury. (Lam et al. 2015) As previously mentioned, platelets play a dynamic role with both the innate and adaptive immune systems in mammals and thrombocytes show similar dynamic parallels in fish. Thrombocytes like platelets have receptors that communicate to other innate immune cells along with molecular machinery for classical and non-classical functions found in mammalian platelets (Table 1.1). Due to their versatile role, platelets and thrombocytes can contribute to both multiple stages of tissue repair, including the induction and resolution of inflammation. Nonetheless, little is known about the role of temperature on the function of this cell type in teleost.

1.9 THE ROLE OF THERMOREGULATION ON THROMBOCYTES AND TISSUE REPAIR

1.9.1 The role of temperature on platelet and thrombocyte activation and function

Although platelets are found to play a multitude of general and specialized functions throughout the mammalian host, little is known about the effects of temperature on platelet function. Limited studies have shown an increase in platelet activation and onset of coagulation pathways in response to increased temperature (Windberger et al. 2020). Similar effects of

temperature can be found in fish where higher rearing temperature of rainbow trout increases thrombocyte count but does not impact their aggregation functions. Another study in channel catfish showed that immunized fish held at a low temperature compared to higher temperatures had lower numbers of critical hematological cell populations including leukocytes and thrombocytes (Martins et al. 2011). While limited, these investigations suggest that an organism's temperature may play a role in thrombocyte count, activation, and function.

1.9.2 The role of temperature on tissue repair

Commonly when one undergoes the swelling (inflammation) of a tissue, they are advised to alternate between cooling and warming the area with the use of heating and cooling packs. This long-standing practice is supported by multiple studies indicating that at a temperature of 30°C-37°C, there is quicker wound healing in humans compared to lower temperatures (Large and Helnbecker1944). Wound temperature was identified as a strong predictor of wound healing outcomes in human patients (Derwin et al. 2023). In two teleost fish, temperature had a greater impact on wound healing than physiological stress (Anderson and Roberts 1975). In salmon, a moderate increase in holding temperature allowed for better outcomes in skin tissue repair (Jensen et al. 2015). Taken together these findings suggest that temperature plays a critical role in tissue repair outcomes in both mammals and teleost suggesting another advantage of the conserved ability to thermoregulate.

1.9.3 Inhibition of the febrile response using anti-pyretics

1.9.3.1 Development of NSAIDs

A long-standing practice in human history has been the search and application of remedies that mitigate maladies such as inflammation, pain, and fever. One of the earliest documentations was in 1763 in which Reverend Edward Stone wrote a letter about the ability of willow bark powder in the treatment of ague (malaria fever) (Wood 2015). After several decades and the improvement of chemical extraction techniques, Raffaele Piria generated salicylic acid from salicin in 1838 and physicians started prescribing these medications for rheumatism (generalized term for inflammation in joints, tendons, ligaments, and bone) (Miner and Hoffhines 2007). In 1897, acetylsalicylic acid, a modified derivative that was more tolerable to the digestive system, became mass produced by the Bayer company (Miner and Hoffhines 2007). After this, aspirin was

widely used for pain. Thus, began the industrial production of aspirin and the development of pharmaceutical NSAID (nonsteroidal anti-inflammatory drugs) production (Hawkey 2005).

1.9.3.2 Current Understanding of NSAID mechanism of action

The current understanding of the mechanisms of NSAIDs is that they inhibit the cyclooxygenase enzyme (COX) (Ghlichloo and Gerriets 2023). Following injury, arachidonic acid, a polyunsaturated fatty acid found on the surface of most cell membranes, is released (Higgins and Lees 1984). Arachidonic acid (AA) is produced through two main pathways: from phospholipids by phospholipases A2 (PLA2) and from 2-arachidonicglycerol (2-AG) converted to AA through monoacylglycerol lipase (MAGL) (Pils et al. 2021). Released arachidonic acid is converted to prostanoids: thromboxanes, prostaglandins and prostacyclin.

Thromboxanes are lipids that allow production of clots via platelet aggregation and vasoconstriction of vessels, prostaglandins (PGE_2) play a role in regulating immune responses, blood pressure, pain and fever, and prostacyclin (PGI_2), which functions as a vasodilator, inhibitor of platelet aggregation and leukocyte adhesion that is partly responsible (in combination with PGE_2) in pain symptoms (Ricciotti and FitzGerald 2011).

The two distinct isoforms of the cyclooxygenase enzyme include COX1 and COX2 (Smith et al. 2000). While both are involved in production of prostaglandins, COX1 is primarily involved in tissue maintenance functions throughout the body, neuroinflammatory functions within the brain, and maintenance of gastric mucosal integrity (Ricciotti and FitzGerald 2011). Interestingly, COX-1 has also been shown to affect platelet aggregation. In contrast, COX2, unlike COX1, is induced by inflammatory stimuli, and results in the downstream mediation of pain, and promotion of acute inflammation (Choi et al. 2009). From the distinct differences between the two isoforms came the development of selective and nonselective NSAIDs, where non-selective NSAIDs inhibit both COX-1 and COX-2 while selective NSAIDs inhibit either COX-1 or COX-2 (Ghlichloo and Gerriets 2023). The selective inhibition of COX-2 was brought on as a response to mitigate symptoms of swelling, redness, and pain.

Frequently utilized non-selective NSAIDs include ibuprofen, diclofenac, naproxen and ketorolac and the most frequently utilized COX-2 selective NSAIDs include celecoxib, rofecoxib, and valdecoxib (Ghlichloo and Gerriets 2023).

1.9.3.3 Ketorolac as the representative NSAID

Unlike ibuprofen and naproxen, ketorolac is an NSAID that is not available over the counter and is often used within hospital inpatient and outpatient settings for pain management in deep muscle pain (Litvak and McEvoy 1990). Unlike other NSAIDs, ketorolac has multiple options for administration including IV (intravenous), IM (intramuscular), oral, and intranasal (Vacha et al. 2015). While it functions the same as other non-selective NSAIDs in inhibiting COX-1 and COX-2, a much smaller dose of ketorolac is just as functional in pain management and fever reduction compared to other non-selective NSAIDs (Ghlichloo and Gerriets 2023).

The recommended IV and IM dose for ketorolac is 0.5mg/kg every 6 hours, after which the circulating medication is below the therapeutic dose (Mahmoodi and Kim 2020). This dosing regimen has previously been used in rainbow trout and goldfish and was well tolerated (Chatigny et al. 2018; Haddad et a.l 2023). After IM injection, ketorolac takes roughly 20 minutes to accumulate in the circulation and function at the therapeutic dose, however, the pharmacokinetic diffusion of ketorolac is subject to change depending on the route of administration and patient specific factors such as blood pressure, heart rate and muscle density. Within the 6 hour therapeutic window, the peak analgesic effects of ketorolac are seen between 1.25 and 4 hours post injection (Mahmoodi and Kim 2020).

1.9.3.4 Known effects of antipyretics on immune system and wound healing

The overwhelming use of antipyretics in response to pain from tissue injury has allowed for some investigation on the effects of antipyretics on the immune system and tissue repair. Numerous fundings indicate the potential detrimental effects of NSAID usage in the tissue repair process. NSAIDs have been demonstrated to have an anti-proliferative effect on fibroblasts, a key cell population in tissue repair (Krischak et al. 2007). The decreased fibroblast population and altered fibroblast function has been shown to result in hypertrophic scar formation, an indication of improper tissue remodelling (Su et al. 2010). The anti-proliferative effect of NSAIDs results in delayed epithelialization and angiogenesis within the early phases of tissue repair and has been

studied in multiple commonly used NSAIDs. Additionally, there are indications of dysregulation of key cytokines such as TGF-β, which plays a prominent role in multiple stages of tissue repair (Su et al. 2010).

Furthermore, key anti-microbial functions such as NO release by innate immune cells is altered by NSAIDs (Schwentker et al. 2002). Nitric oxide plays roles in inflammation, angiogenesis and cell proliferation, all critical phases of tissue repair. Patients administered NSAIDs have shown a marked decrease in NO production, resulting in delayed or improper wound healing (Zhao-Fleming et al. 2018). Chronic administration of ketorolac to wounded rats during tissue repair resulted in decreased tensile strength of wounds and decreased collagen concentration (Haws et al. 1996).

While NSAIDs provide substantial relief from pain and swelling post injury, there are indications of NSAID interference with critical components of the tissue repair process. Furthermore, fever may allow for improved outcomes in tissue repair on the cellular and molecular level, and inhibition of fever may be detrimental in the tissue repair process.

1.9.4 Summary

Acute inflammation is a critical process in host defense against internal and external pathogens. Inflammation has been studied since ancient times and heat is considered a cardinal sign. However, there is still standing debate on if the rising of core body temperature, fever, has net negative or net positive effects on host health and immune responses. My lab has worked in developing an ectothermic model of fever in which a goldfish can exert a natural behavioral febrile response and has characterized changes in effector cell recruitment and function in response to immune challenge. Furthermore, we have previously demonstrated the altered recruitment and function of key effector cells in response to a natural fever after immune challenge. Not only were effector cell phenotypes and functions different before and after the peak of leukocyte infiltration, but these functions were significantly altered when a fish was allowed to exert a febrile response.

As changes in leukocyte kinetics and function were reported in response to a zymosan-induced acute inflammatory response in response to fever, I was curious to determine the impact on a dynamic cell population, thrombocytes, whose mammalian equivalent (platelets) have been

demonstrated to have immunomodulatory effects in response to various stimuli, including temperature. Therefore, my project involved the characterization of goldfish thrombocytes in acute inflammation. While thrombocytes have been reported in many teleost models, including goldfish, thrombocytes within the site of immune challenge have not been quantified. In the limited studies on goldfish thrombocytes, thrombocytes show capacity to preferentially phagocytose smaller particles. Multiple other studies in teleost show thrombocyte capacity to act as antigen presenting cells and detect PAMPs with the use of MHCII and TLRs. These findings and others indicate that the immunomodulatory functions of platelets may be long conserved from thrombocytes. However, due to limited available reagents in the quantification of thrombocytes, and thrombocytes being significantly lower in proportion than other leukocyte subsets, the kinetics of thrombocyte proliferation and activation have not been well established. Therefore, an aim of my project was the characterization of thrombocytes within the site of challenge with the use of cytochemical staining, molecular quantification, and high throughput flow cytometry. As a natural febrile response allowed for earlier and greater leukocytic recruitment, I hypothesized that a natural febrile response would allow for an earlier and greater thrombocyte recruitment as well.

Platelets are classically known to be involved in hemostasis and thrombus formation. Over the last three decades platelets have been observed to play roles within other processes in restoring hemostasis, namely, tissue repair. My lab has utilized a cutaneous infection model using *A. veronii* and examined pathogen clearance and tissue repair in multiple temperature conditions. Using this model, my lab has demonstrated a greater pathogen clearance at higher temperatures and at dynamic temperatures. Furthermore, goldfish hypothalamic increase of critical pro-inflammatory genes and anti-inflammatory genes are heightened in dynamic temperature conditions. Dynamic temperature conditions have also allowed for faster wound closure compared to static temperature conditions. As fever was demonstrated to have enhancing effects on tissue repair and pathogen clearance, I was curious to examine the impact of fever and fever-inhibition on thrombocyte proliferation and activation. I hypothesized that there would be greater recruitment of thrombocytes in dynamic temperature conditions compared to fever-inhibited conditions and static temperature conditions. However, because goldfish thrombocyte functions aren't well established, the implications of a greater thrombocyte count versus a lower thrombocyte count were unknown.

Therefore, to address this, I aimed to quantify thrombocyte proliferation and activation, and then contextualize this by examining the pro- and anti-inflammatory cytokines in furuncles. Furthermore, I examined the tissue histology of furuncles to then determine the impact of fever and fever-inhibition on tissue repair at the molecular and tissue level.

In summary, my thesis focusses on the impact of fever on thrombocytes in acute inflammation and restoration of homeostasis. Using a self resolving peritonitis model and a cutaneous wound model, I examined thrombocytes and the effects of ketorolac induced fever-inhibition. These findings shed some light on the potential immune enhancing benefits of the febrile response and show altered kinetics of a dynamic cell population, thrombocytes.

Chapter 2: Materials and Methods

2.1 Animals

2.1.1 Goldfish

The goldfish (*Carassius auratus*) used in all studies were acquired from aquatic imports. The fish were 15cm in length and weighed roughly 20 grams. The goldfish were housed in the aquatics facility in the Biological Sciences Building at the University of Alberta. The goldfish were held at 16℃ in opaque tanks in dechlorinated and oxygenated water. Goldfish were given at least 14 days to acclimatize before use in experiments. When using for experiments, fish were netted and anesthetized using 50mg/L tricaine methane sulfonate (TMS). Once the fish were anesthetized, fish were sacrificed via cervical dislocation to minimize unnecessary stress to the animals. All handling and sacrificing procedures were completed in conjunction with the Animal Care and Use Committees' and Science and Animal Support Services' procedures and protocols.

2.2 Immune Challenges

2.2.1 Intraperitoneal Injections with Zymosan

Goldfish that were 15-20cm were anesthetized in a TMS solution. Fish were injected using 21G needles loaded with 100µL of Zymosan A (Cayman Chemicals) solution at 25µg/mL. Zymosan was isolated from *Saccharomyces cerevisiae*. Fish were injected 1-2cm below the pectoral fins in the soft tissue above the anus at a 45˚ angle. After injection, fish were returned to their original aesthetic-free water which was actively oxygenated and placed in either the ATPT tank or the fixed 16℃ tanks once awake.

2.2.2 Peritonitis Infection with *Aeromonas veronii*

2.2.2.1 Culturing Aeromonas veronii

Aeromonas Veronii biovar sobria cultures were prepared by the addition of previous stock solution of *A. veronii bv sobria* to sterile, autoclaved tryptic soy broth (TSB) and left overnight on a shaker at room temperature until the Optical Density (600nm) was between 1-1.2 (indicating growth in log phase).

2.2.2.2 Creation of an Aeromonas Veronii infected wound site

Fish were anesthetized in 50mg/L TMS. Fish were placed on a benchkoat surface, and a 4 by 4 patch of scales was removed below the middle of the dorsal fin, 1cm above the midline. Three microincisions were made and 10µL of actively growing *A. veronii* was swabbed on the microincisions. Fish were then placed in their original anesthesia-free water with a bubbler. Once awake, fish were placed in either the fixed 16℃ tanks or the ATPT tank for a period of days.

2.3 Temperature conditions

2.3.1 Static Temperature Tank

As my research required the measurement of a dynamic behavioral fever compared to a static temperature range, two different tanks were used. Fish were housed in static conditions, in tanks where water temperature was held consistently at 16℃. While goldfish have a large thermal range, the fish housed within the Aquatics facility at the University of Alberta were acclimatized at 16℃.

2.3.2 Annular Thermal Preference Tank

To determine the potential benefit of host-driven thermoregulation, in my thesis project, I utilized a behavioral fever model. In order to allow goldfish to exhibit natural behavioral thermoregulation, I used an Annular Thermal Preference Tank (ATPT) (Haddad et al. 2023). The ATPT allows a fish to swim to a preferred temperature zone, and exhibit thermal preference. The temperatures of the ATPT range from 16℃ to 26℃ with 8 thermal zones from the highest and lowest temperature. Additionally, to limit stress in the behavioral response, there is a stable gradient between each temperature zone. In order to map behaviors such as lethargy, thermal preference and mean zone transitions, we utilize a high-resolution camera and tracking software to record thermoregulatory patterns over long observation times.

2.3 Administration of Ketorolac

2.3.1 Intraperitoneal Injection with Ketorolac

To evaluate the effects of fever inhibition on thrombocyte activation, peritoneal injections of ketorolac were used. Ketorolac tromethamine (Toradol IM) at 10mg/mL solution was diluted to 0.5mg/kg and 100µL are injected using a 21G needle into the peritoneal cavity of goldfish

once fish were anesthetized in TMS. Fish were injected 1-2cm above the anus below the pectoral fins in the soft tissue at a 45˚ angle. Fish were returned to their anesthetic free tank water and a bubbler was added while fish were transported to the ATPT tank.

2.4 Isolation of Peritoneal Leukocytes

2.4.1 Intraperitoneal Lavage Using Heparinized PBS

After a set number of hours post intraperitoneal injection with zymosan, fish were collected from their tanks and anesthetized in TMS. A cervical dislocation was preformed and any remaining blood on the fish was wiped off. The peritoneal cavity was opened from the dorsal fin to above the midline such that the majority of the peritoneum was visible after opening up the window. The peritoneal cavity was lavaged with 10mL of sterile 1X PBS $^{-/-}$ mixed with 50U/mL heparin in syringes with a 21G needle and the PBS solution with peritoneal contents was collected in 50mL conical tubes and placed on ice while awaiting further processing.

2.4.2 Enumerating peritoneal leukocytes.

Leukocytes in the peritoneal lavage (10mL) were enumerated using a hemacytometer (Fisher) and visualized on a light microscope (Nikon Eclipse TS100) at 10X. Cell concentration was calculated using the formula below:

Cell concentration = (# of cells counted)/ (# of outer squares counted) x Dilution factor x 10^5

2.5 Leukocyte Bioassays for Imaging Flow Cytometry

2.5.1 Reactive Oxygen Species and Nitric Oxide Assay

Once cells were harvested and enumerated, an aliquot containing $2x10^6$ cells was centrifuged at 1350 RPM for 8 minutes at 4°C. The supernatant was discarded, and cells were resuspended 1.5mL of 1X PBS $^{-/-}$ and washed again (1350 RPM for 8 minutes at 4°C). The supernatant was discarded, and cells were resuspended in 500µL of serum-free MGFL in a 2mL FACS tube. Once resuspended, 0.5µL of CellRox^{TM} Deep Red Reagent (Invitrogen) and 1µL of 1:10 diluted DAF-FM Diacetate (Invitrogen) in 1X PBS$^{-/-}$ was added to the cells. Cells were then incubated for 30 minutes on ice in the dark. After incubation, 2mL of 1X PBS$^{-/-}$ was added to the cells and cells were washed twice (1350 RPM for 8 minutes at 4°C). After two washes, the supernatant was discarded, and cells were resuspended in 140µL of 2% formaldehyde and

incubated at 4°C in the dark for a minimum of 10 minutes. Once ready for use, cells were centrifuged at 1350 RPM for 8 minutes at 4°C and the supernatant was discarded prior to flow cytometric analysis.

Imaging flow cytometry data was acquired on the IS100 Imagestream (Amnis Corporation) and analyzed using the ISX software (Amnis Corporation).

2.5.2 Monoclonal Anti-Thrombocyte Antibody Assay

Leukocytes were collected, enumerated, and placed on ice. An aliquot of $2x10^6$ cells was centrifuged at 350 x *g* for 8 minutes at 4°C, the supernatant was discarded, and cells were resuspended in 2mL of serum free MGFL media. Resuspended cells were then transferred to a 2mL FACS tube, and 200µL of the anti-thrombocyte monoclonal primary antibody was added to the cells. Cells were incubated on ice and in the dark for 2 hours. After incubation 2mL of 1X $PBS^{-/-}$ was added to the cells and cells were washed (350 x *g* for 8 minutes at 4°C). The cells were then resuspended in 2mL of serum free MGFL media, 4µL of PE anti-Rat IgG2a (Biolegend) secondary antibody was added and incubated for 2 hours on ice. After incubation in secondary antibody, 2mL of 1X $PBS^{-/-}$ was added to the cells and cells were washed (350 x *g* for 8 minutes at 4°C). The supernatant was discarded prior to cells being used in imaging flow cytometry.

Imaging flow cytometry data was acquired on the IS100 Imagestream (Amnis Corporation) and analyzed using the ISX software (Amnis Corporation).

2.6 Histological Staining

2.6.1 Cytochemical Staining

2.6.1.1 Sudan Black B Staining

Glutaraldehyde fixative solution was prepared by the addition of 25mL of acetone to 75mL of Glutaraldehyde solution (Sigma Aldrich). One hundred thousand cells from peritoneal lavages were centrifuged at 55 x *g* for 6 minutes onto microscope slides using a cytocentrifuge (Eppendorf). Cells were then fixed on slides by incubation of cells in Glutaraldehyde Fixative solution for 1 minute at 4°C. Cells were rinsed in distilled water and were incubated in Sudan Black B Solution (Sigma-Aldrich) in a Coplin jar for 5 minutes with agitation. Excess Sudan Black B solution was removed via three washes in ethanol (70%) until there was no black runoff

from slides. Slides were then rinsed in distilled water to remove excess ethanol. Slides were incubated in Hematoxylin Solution Gill No. 3 (Sigma Aldrich) a Coplin jar for five minutes. Excess reagent was rinsed off thoroughly with tap water and slides were left to air dry. After air drying, slides were mounted with Toluene (Sigma Aldrich) and a slide cover was added.

2.6.1.2 Hema 3

One hundred thousand cells were spun onto slides at 55 x *g* for 6 minutes using a cytocentrifuge. Cells were fixed by Hema3 Fixative (Fisher Scientific). Slides were tapped to remove excess solution and incubated in Hema3 Solution 1(Fisher Scientific) for 1 min and excess was blotted off. Slides were then counterstained with Hema3 Solution II (Fisher Scientific) for 30 seconds, rinsed with tap water, and mounted using Permount (Fisher Scientific) once dry.

2.6.1.3 Periodic Acid Schiff (PAS) Stain

One hundred thousand cells were centrifuged at 55 x g for 6 minutes in a cytocentrifuge. Slides were then fixed in an ethanol-formaldehyde solution. Ethanol-formaldehyde solution was made fresh every use by the addition of 5mL formaldehyde (Fisher Scientific) and 45mL ethanol (95%) . After fixing slides were rinsed in tap water for 1 minute. Slides were then immersed in Periodic Acid Solution (Sigma Aldrich) at room temperature for 5 minutes. After incubation, slides were washed in distilled water five times. Slides were incubated in Schiff's Reagent (Sigma Aldrich) for 15 minutes at room temperature. After incubation, slides were washed in distilled water. Slides were counterstained in Hematoxylin Solution Gill no. 3 (Sigma Aldrich) for 90 seconds at room temperature. Slides were then rinsed in distilled water and left to air-dry. Once dry, cells were mounted with Permount (Fisher Scientific) and left to dry prior to viewing on the DM1000 microscope (Leica).

2.6.2 Histological Staining of Tissues

2.6.2.1 Fixing and Paraffinizing

Furuncles, when collected, were immediately preserved in formalin (Fisher). All samples were dehydrated with serial decreasing concentrations of ethanol and then paraffinized in the Microscopy Facility (Biological Sciences, University of Alberta, Alberta). Samples were all loaded and then dehydrated and paraffinized by an Automatic Benchtop Tissue Processor TP1020 (Leica).

2.6.2.2 Masson's Trichrome stain

After paraffinization, thin sections of samples were acquired on microscope slides, and in the fume hood, slides were immersed in Toluene (Fisher) twice for 5 minutes. Samples were then immersed into serial dilutions (100%, 90%, 70% 50%) of ethanol for 2 mins each. To stain the nuclei, Hematoxylin Gill III (Surgipath) were used for 1.5 minutes. After staining nuclei, slides were washed well in cold running tap water for 10 minutes, and then under distilled water for 1 minute. Slides were then stained for 2 minutes Ponceau-Acid-Fuchsin solution prepared by mixing the components in Table 2.3. Samples were rinsed in distilled water for 1 minute, then differentiated for 5 minutes in 1% phosphomolybdic acid (Fisher) and immediately transferred to Acetic Aniline Blue solution for 3 minutes. Slides were then rinsed in distilled water for 1 min and immersed in fresh phosphomolybdic acid (Fisher) for 5 minutes. Slides were placed in 1% aqueous acetic acid (Fisher) for 3 minutes and serially dehydrated in 95%, 100% and 100% ethanol for 2 minutes each. Slides were then immersed in toluene for 2 minutes and mounted with DPX mounting medium (Electron Microscopy Sciences), covered with coverslips and left to dry before visualizing.

2.6.2.3 Hematoxylin and Eosin Stain

After paraffinizing and thin sectioning samples onto slides, slides were immersed in fresh Toluene (Fisher) for 2 minutes and then 8 minutes. Slides were then serially diluted in ethanol (100%, 100%, 90%, 70% and 50%) for 2 minutes and then placed in distilled water for 2 minutes. Slides were then placed in Hematoxylin Gill no. 3 (Surgipath) for 2 minutes and then rinsed in distilled water followed by cold tap water for 15 minutes. Slides were then washed with ethanol (70%) for 2 minutes prior to a 30 second incubation in Eosin (Surgipath). Slides were then washed with fresh ethanol (100%) twice for 2 minutes and then placed in toluene for 2 minutes. Immediately after removing the slides from toluene they were mounted with coverslips using DPX mounting medium (Electron Microscopy Sciences).

2.7 Quantitative PCR

2.7.1 RNA Extraction

Samples were thawed from -80°C on ice and placed in 12mL round bottom tubes. TRIzol (Ambion) was added based on the tissue (Furuncle 1.5mL, kidney 1mL, peritoneal lavage 1mL).

Tissue was then homogenized and 1mL of the TRIzol mixture was added to a 2mL microcentrifuge tube and left undisturbed for 5 minutes. Two hundred microlitres of 1-bromo-3-chloropropane (BCP) (Sigma Aldrich) was added to TRIzol mixture and vortexed well. Tubes were left undisturbed for 3 minutes at room temperature prior to centrifugation at 12 000 x g for 15 minutes at 4°C. The aqueous phase was placed into a fresh 2mL microcentrifuge tube without disturbing the debris and organic phases. To the aqueous phase, 500µL of ice-cold isopropanol and 1µL of glycogen (Roche) was added and mixed by inversion. Tubes were stored in -80°C for a minimum of 4 hours.

Tubes were removed from -80°C and cooled on ice before centrifugation at 12 000 x g for 10 minutes at 4°C. The supernatant was discarded and 1mL of cold ethanol (75%) was added. Samples were centrifuged at 7500 x g for 5 minutes at 4°C, and the supernatant was discarded. Samples were centrifuged at 7500 x g for 5 minutes at 4°C and the supernatant was discarded. Tubes were blotted on paper towel and left to air dry for 10 minutes. After drying, 11µL of nuclease free, RNAse free H_2O was added, and RNA pellet and heat shocked at 56°C for 10 minutes.

The nucleic acid concentration was quantified using a Nanodrop apparatus at an absorbance of 260nm. Samples were read at 230nm and 280nm to determine potential phenolic and protein contaminations from the organic phase.

2.7.2 cDNA generation

RNA concentration determined by the Nanodrop apparatus, was diluted to 1µg/10µL using Nuclease Free H_2O. All cDNA was generated using a High-Capacity cDNA Reverse Transcription Kit (Biolegend). A 2X RT Master Mix was prepared (Table 2.1) and 10µL of the RT Mastermix was added to 10µL of the diluted RNA. Tubes were all kept on ice until they were added to the Mastercycler (Eppendorf). cDNA was then synthesized by running the thermal cycler at the manufacturer's specifications (Table 2.2).

2.7.3 Primers

All cytokine primers utilized in my thesis were previously validated for use in goldfish. The *c-mpl* gene sequence is not yet annotated in the goldfish genome however, a primer sequence has previously been reported by Katakura et al (2015). All other primers listed in Table

2.9 have been previously validated (Haddad et al. 2023; Grayfer et al. 2008; Grayfer & Blosevic 2009). All primer sequences are listed in Table 2.9.

Primers were solubilized using Nuclease Free H_2O as per manufacturer's guidelines.

2.7.4 qPCR Assembly

qPCR Mastermix was created (Table 2.10) and added to 384-well qPCR plates on ice prior to cDNA addition. cDNA was added at 1:100 dilutions for reference genes (β-actin) and 1:10 dilutions for target genes (*cmpl, tnfa, tgfb, il10, il1b*). Total volume per well was 10 µL. Samples were run in triplicate and cycled 40 times. Each cycle consisted of 60s at 60℃ and 15s at 95℃ after an initial 10-minute heating period of 95℃. qPCR was preformed using the QuantStudio 6 Real-Time PCR System (Applied Biosystems) in which RQ values were normalized against baseline gene expression of day 0 untreated fish. β-actin was used as a reference gene and relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ quantification method.

2.8 Medias

2.8.1 Phosphate Buffered Saline (10X)

Multiple protocols in this thesis required the use of 1X PBS, diluted from 10X PBS. The 10X PBS was made by combining the components in their respective quantities described in Table 2.5. After completely dissolving all components to a homogenous mixture, the 10X PBS was filtered using a 22µm filter and autoclaved to remove any additional contaminants.

A 1 in 10 dilution using filter sterile, autoclaved milliQ water, to generate 1X PBS used in protocols.

2.8.2 Resuspension media

During long incubation steps with antibodies and fluorophores, cells were placed in serum-free MGFL medium. MGFL medium was created by combining the components in table 2.6, which required 10X HBSS and Nucleic Acid Precursor solution which were made prior by ingredients and volumes listed in table 2.7 and 2.8 respectively. The media was then filtered through a Steritop Filter unit (Millipore) and stored at 4℃.

2.9 Overall Experimental Design

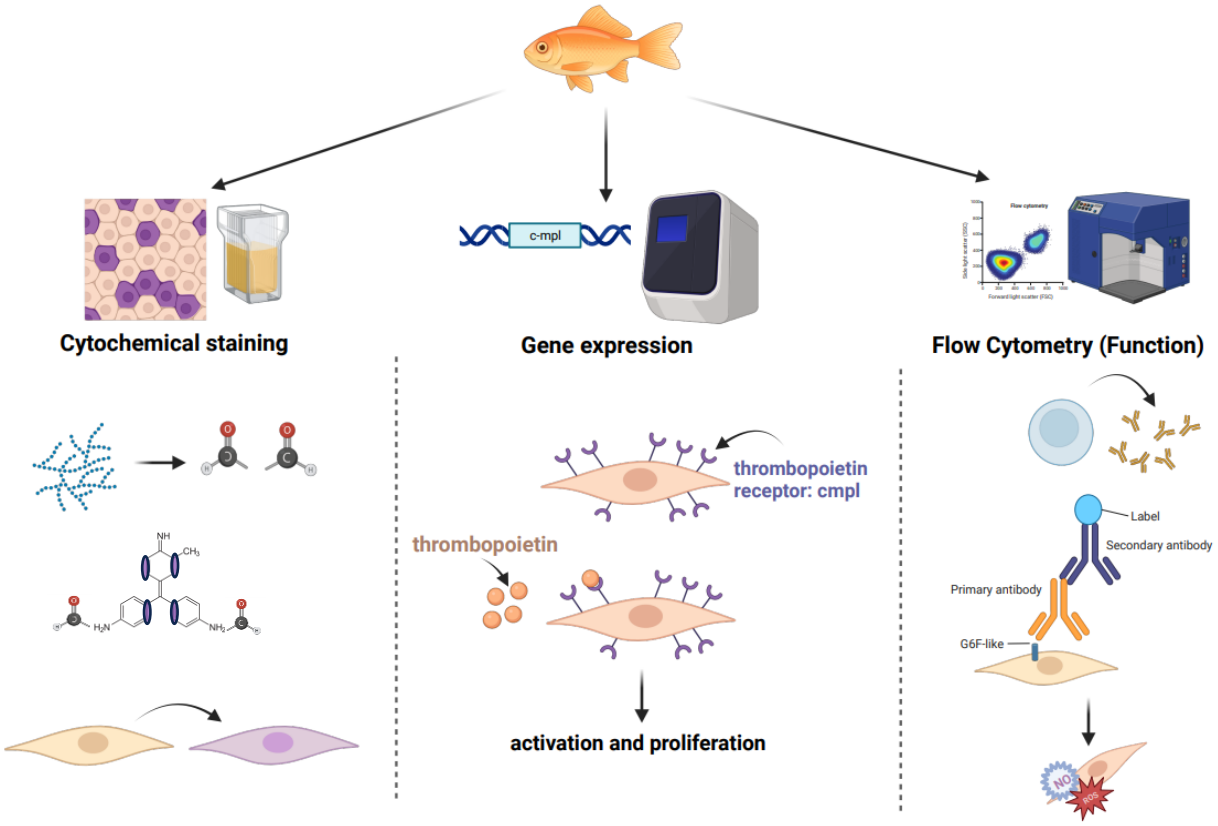


Figure 2.9.1 Methods Summary for Characterization of Thrombocytes

Cytochemical staining with the Periodic Acid-Schiff stain was used to separate thrombocyte and erythrocyte cell populations. Glycogen within thrombocytes is cleaved to aldehydes using a periodic acid, and aldehydes are stained purple with the Schiff's reagent rendering thrombocytes a purple cytoplasm. Molecular quantification of thrombocytes was achieved through measurement of fold change on thrombopoietin receptor (*cmpl*), a marker for thrombocyte activation and proliferation. RNA was extracted, and qPCR was completed to measure *cmpl* of kidneys and furuncles of fish. Functional characteristics of thrombocytes are assessed with imaging flow-cytometry by staining with a primary monoclonal antibody that binds to the G6F-like component of thrombocyte cell membrane. Further assays can be completed for additional functions such as release of antimicrobial molecules such as ROS and NO.

TABLES

Table 2.1 Composition of RT Mastermix for cDNA

Component	**Volume**
10X RT Buffer	2.0 µL
25X dNTP Mix (100mM)	0.8 µL
10X RT Random Primers	2.0 µL
MultiScribe Reverse Transcriptase	1.0 µL
Nuclease-free H_2O	4.2 µL

Table 2.2 PCR Thermal Cycler Specifications

Settings	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Time	10 minutes	120 minutes	5 minutes	HOLD
Temperature	25°C	37°C	85°C	4°C

Table 2.3: Components of Ponceau-Acid-Fuchsin Solution

Component	**Amount**	**Source/Cat#**
Acid Fuchsin	0.6 g	Fisher/ F-97
Ponceau de xylidine	1.4 g	Michrome/565
Distilled Water	198 mL	-
Glacial Acetic Acid	2mL	Fisher/A38-212

Table 2.4: Components of Acetic Aniline Blue

Component	Amount	Source/Cat#
Aniline Blue	4g	Fisher/A967
Distilled Water	196mL	-
Glacial Acetic Acid	4mL	Fisher/A38-212

Table 2.5: Components of 10X Phosphate Buffered Saline

Component	**Amount**	**Source/CAS#**
KCl	2.0g	Fisher/7447-40-7
KH_2PO_4	2.0g	Fisher/7778-77-0
NaCl	80.0g	Millipore Sigma/7647-14-5
Na_2HPO_4	11.44g	Millipore Sigma/7558-79-4

Table 2.6: Components of MGFL-15 Medium (Serum-free)

Component	**Amount**	**Source/CAS#/Cat#**
HEPES	7.0g	Invitrogen/7365-45-9
KH_2PO_4	0.688g	Fisher/7778-77-0

K_2HPO_4	0.570g	Millipore Sigma/7758-11-4
NaOH	0.75g	Invitrogen/1310-73-2
$NaHCO_3$	0.34g	Millipore Sigma/144-55-8
10x HBSS *	80mL	
MEM Amino Acid Solution (50X)	25mL	ThermoFisherSci/11130051
MEM Non-essential Amino Acid Solution (100X, 10mM)	25mL	ThermoFisherSci/11140050
MEM vitamin solution	20mL	ThermoFisherSci/11120037
Sodium pyruvate solution (100mM)	25mL	ThermoFisherSci/11360070
Nucleic Acid Precursor Solution *	20mL	
L-glutamine (200mM)	0.5844g	ThermoFisherSci/A2916801
Insulin	0.01g	Invitrogen/RP-10908
GFL-15	1L	Invitrogen/31415029
Beta-mercaptoethanol	7µL	Millipore Sigma 60-24-2
Autoclaved Filter Sterile H_2O	2L	

Table 2.7: Components of 10X Hanks Solution (HBSS)

Component	Amount	Source/CAS#
KCl	2g	Fisher/7447-40-7
KH_2PO_4	0.3g	Fisher/7778-77-0
NaCl	40g	Millipore Sigma/7647-14-5
$Na_2HPO_4{\cdot}7H_2O$	0.45g	Sigma Aldrich/ 7782-85-6
D-glucose	5g	Sigma Aldrich/ 50-99-7
Phenol red	0.05g	Millipore Sigma/ 143-74-8
Autoclaved Filter Sterile H_2O	500mL	

Table 2.8: Components of Nucleic Acid Precursor Solution

Component	Amount	Source/Cat#
Adenosine	0.067g	ThermoFisherSci/A10781.09
Cytidine	0.061g	ThermoFisherSci/111810500
Hypoxanthine	0.034g	ThermoFisherSci/A11481.06
Thymidine	0.061g	ThermoFisherSci/A11493.06
Uridine	0.061g	ThermoFisherSci/A15227.06
Autoclaved Filter Sterile H_2O	100mL	

Table 2.9 Primer Sequences for Quantitative PCR

Target Gene	Abbreviation	Sequence 5'-3'
Beta Actin	B-actin FW	GAC CAA CCC AAA CCT CTC AA
	B-actin RV	AGT CAA TGC GCC AAA CAG A
Tumor Necrosis Factor alpha	tnfa FW	TCA TTC CTT ACG ACG GCA TTT
	tnfa RV	CAG TCA CGT CAG CCT TGC AG
Interleukin 1 beta	il1b FW	GAT GCG CTG CTC AGC TTC T

	il1b RV	AGT GGG TGC TAC ATT AAC CAT ACG
Transforming Growth Factor Beta	tgfb FW	GTA CAC TAC GGC GGA GGA TTG
	tgfb RV	CGC TTC GAT TCG CTT TCT CT
Interleukin 10	il10 FW	CAA GGA GCT CCG TTC TGC AT
	il10 RV	TCG AGT AAT GGT GCC AAG TCA TCA
Thrombopoietin receptor	cmpl FW	AGA ATG GAG TGA CTG GTC GAA AC
	cmpl RV	GAT GAG CAG AGC CAC TGG AATA

Table 2.10 qPCR Master Mix Ratio per well

Reagent	**Quantity**
SybrGreen	5 µL
Nuclease-Free H_2O	1.5 µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
cDNA	2.5 µL

Chapter 3: Behavioral fever is a robust regulator of functional innate immune responses.

3.0 Introduction

It has been well established that fever is a hallmark of acute inflammation and acute inflammation is often in response to the host encountering microbial challenges including bacteria, viruses, parasites, and fungi. Fever has been conserved throughout evolution for millions of years, while endotherms partake in a metabolic fever and internally raise core body temperature, ectotherms relocate to alter core body temperature (John Victor Hurley 1972; Kluger 1979; Terrien et al. 2011; Garami et al. 2018). Endotherms also undergo a metabolic fever which includes several metabolic pathways to increase core body temperature including shivering for thermogenesis and reduction of heat loss (Terrien et al. 2011; Roth and Blatteis 2014; Garami et al. 2018). Ectotherms do not undergo shivering or upregulation of the same metabolic pathways in an endothermic metabolic fever, and instead undergo a behavioral fever response via relocation to warmer and cooler environments for thermoregulation (Evans et al. 2015; Kluger 1979). Therefore, the study of behavioral thermoregulation of ectotherms serves as a strong model in comparative biology to understand an alternative mechanism for the induction of fever. As fever and acute inflammation are heavily interlinked, and fever has been shown to alter outcomes of acute inflammation, the functions of immune cells in the acute inflammatory process can provide important insights into this complex process (Serhan et al. 2020).

Therefore, the first objective of my thesis was to investigate the effects of fever on leukocyte populations during acute inflammation. Previous students in the lab reported changes in leukocyte infiltration throughout the acute inflammatory window (Havixbeck et al. 2015). After zymosan challenge, peak leukocyte infiltration of goldfish held at constant temperature was reported at 18 hpi. Additionally, the prominent population of total leukocytes during the peak of infiltration was neutrophils. Interestingly, two distinct phenotypes of neutrophils were present in the acute inflammatory window (48 hours), the first being pro-inflammatory neutrophils present before peak infiltration and during peak infiltration, and the second being pro-resolving neutrophils present hours after peak infiltration (Havixbeck et al. 2015). Neutrophils isolated in the pro-inflammatory phase released potent lipid mediators (LTB_4) that induced significant functional increases in macrophage anti-microbial responses. Neutrophils

isolated in the resolution phase of acute inflammation released high levels of another potent lipid mediator (Lipoxin A4) and induced functional changes in attenuation of macrophage ROS responses. These investigations highlighted the dynamic role of goldfish neutrophils during acute inflammation, and how this cell population can change phenotypes in response to cues during acute inflammation (Havixbeck and Barreda 2015). Furthermore, it established a foundation for the measurement of cell populations within the acute inflammatory window in goldfish. With the development of the ATPT in our lab, other students have measured functional differences in leukocyte recruitment and noted an earlier infiltration in febrile (dynamic) conditions compared to static (Wong 2017). Additionally, they isolated leukocyte subpopulations and noted some difference in neutrophil, macrophage/monocyte, and lymphocyte populations in static and dynamic conditions (Wong 2017). Interestingly, febrile (dynamic) conditions resulted in a significant drop in neutrophil populations immediately after the peak of infiltration, which was now identified as 16 hours post zymosan challenge. These results demonstrated marked changes in infiltration kinetics in three leukocyte subsets when a fish was allowed to exhibit to natural thermoregulatory response (ie. choose and translocate to preferred temperature).

My next research aim was to determine effector functions resulting from altered cell kinetics in response to fever. Others have shown altering lipid mediators during the acute inflammatory profile in response to zymosan challenge at static temperature and noted marked differences in antimicrobial response profiles (ROS) of cells isolated during different phases of acute inflammation (Havixbeck et al. 2015). After development of the ATPT, an additional antimicrobial response was assessed, NO species production (Wong 2017; Haddad et al. 2023). Marked differences in antimicrobial profiles were observed in response to febrile and afebrile conditions. These significant differences in antimicrobial profiles were also observed in response to other immune challenges such as *A. veronii* (Haddad et al. 2023). Using previously applied methods in the lab, I aimed to first characterize leukocyte infiltration and function within the peritoneum in both temperature conditions and apply these techniques in quantifying a cell population that may have potent immunomodulatory effects within acute inflammation, and the effect of fever on thrombocyte population. Previous collaborative investigations between my lab and others demonstrated the isolation of thrombocytes from total peripheral blood leukocytes and showed that thrombocytes possess phagocytic capacity through imaging flow-cytometric assays (Nagasawa et al. 2014). While this study was substantial in showing a novel thrombocyte

function in teleost, these thrombocytes were harvested from total blood and excluded thrombocytes participating in the site of immune challenge (peritoneum). Therefore, my last aim was to investigate the impact of fever on the infiltration kinetics of thrombocytes in the peritoneum which necessitated multiple methods of quantification including cytochemical staining, gene upregulation, and high throughput imaging flow cytometry.

To understand the impact of behavioral fever on acute inflammation, this chapter focusses on the recruitment [1] and function [2] of essential innate immune effector cell populations in response to zymosan induced peritonitis. To expand on the work of others in my laboratory and others in the field, I characterized the morphology [3] and recruitment [3,4] of another dynamic cell population, thrombocytes. Thrombocytes are the closest evolutionary ancestor to human platelets, which demonstrate dynamic interactions with innate and adaptive immune cells, as well as active involvement in inflammation.

3.1 Fever alters the recruitment kinetics of leukocytes in goldfish challenged with zymosan.

Ectothermic animals will seek warmer temperature environments to thermoregulate, and the conserved response of thermoregulation provides a strong argument for the beneficial effects of a fever (Evans et al. 2015; Haddad et al. 2023). Others have previously established that a fever allows for enhanced pathogen clearance but the regulatory mechanisms controlling this process have only recently been examined (Haddad et al. 2023; Havixbeck et al. 2015). Leukocytes are a heterogenous population of cells of the innate immune system that carry out immunomodulatory and effector functions for pathogen clearance. To derive the effect of a natural fever, I began to look at leukocyte recruitment post injection with zymosan, within the first 24 hours of injection when fish were allowed to exhibit a behavioral fever response compared to fish held at a static temperature. The fish allowed to exhibit a natural fever response were placed in the ATPT tank (herein referred to as T_D or dynamic fish), while fish held at a static temperature were placed in a standard holding tank held at 16ºC (herein referred to as T_S or static fish) for 24 hours. Fish were collected every 4 hours in the 24-hour observation period. Leukocytes were isolated from the peritoneal cavity via a peritoneal lavage and enumerated for each time point (**Figure 3.2.1**). The peak of leukocyte infiltration in static and dynamic conditions occurred at 16 hpi with a mean of $92 \times 10^5 \pm 9.01$ leukocytes in T_S fish, and a mean $125 \times 10^5 \pm 9.01$ leukocytes in T_D fish. Infiltration profiles in both static and dynamic conditions showed a curve-like distribution

peaking at 16 hpi, however the number of cells in the peritoneal cavity before and after the peak showed significant differences. In static conditions, T_S at 12 hpi showed lower average total leukocytes ($59.6 \times 10^5 \pm 7.29, p < 0.05$) than T_D fish ($88.8 \times 10^5 \pm 7.29, p < 0.05$) . At 20 hpi, T_S fish showed higher total leukocytes ($70.0 \times 10^5 \pm 10.5, p < 0.05$) compared to T_D fish ($24.8 \times 10^5 \pm 10.5, p < 0.05$), and this trend continued for every time point up to 48 hpi. At 20 hpi there is a significant drop in total leukocytes in TD fish and by 48 hpi, total leukocyte count returns to baseline conditions ($8.40 \times 10^5 \pm 5.89, p < 0.01$). In comparison, in T_S fish, total leukocyte count does not return to baseline levels even up to 48 hpi ($49.6 \times 10^5 \pm 5.89, p < 0.01$). Overall, these findings indicate a difference in recruitment kinetics depending on if the fish was allowed to exhibit a natural behavioral fever post immune challenge with zymosan.

3.2 Fever alters the kinetics of neutrophils, macrophage/monocytes and lymphocytes in acute inflammation.

In murine and mammalian models there is a substantial recruitment of both macrophages and neutrophils to the site of infection, in fish, neutrophils have been identified as the more predominant effector, while circulating macrophages make up a slightly smaller proportion of effector phagocytes (Davies et al. 2011; Havixbeck et al. 2015; Havixbeck and Barreda 2015). While the recruitment of total leukocytes was different between fish allowed to exhibit a natural behavioral fever and fish held at a constant temperature, total leukocyte count does not specifically identify the leukocyte subsets within the peritoneal cavity. Therefore, to identify which cell subsets and their proportion relative to other leukocyte subsets, I visualized and enumerated three cell populations that make up most of the infiltrating innate immune cells in goldfish. These populations are macrophage/monocytes, neutrophils, and lymphocytes. Unlike murine innate immune cells, goldfish macrophages, monocytes, and neutrophils show similar morphologies under the microscope and therefore the Sudan Black B stain was used to distinguish between the neutrophil and macrophage/monocyte populations (Blumenreich 2011). Sudan Black B stains the granules within neutrophils, and the absence of acidic granules in macrophages and monocytes allows for the resolution of both populations (Cornbleet 1998; Haddad et al. 2023). Lymphocytes and macrophages differ in nuclear and cytoplasmic

morphology and therefore a Wright-Giemsa stain allowed for their identification (Witeska et al. 2022).

When total leukocytes were separated and enumerated into subsets, there were key differences in the recruitment of each leukocyte subset between fish held at static and dynamic temperatures. Of the total leukocytes infiltrating the peritoneal cavity, neutrophils made up most of the phagocytosing cells (**Fig 3.2.2**). The neutrophil population in T_D fish infiltrated as early as 4 hpi ($47.1 \times 10^5 \pm 5.27$), peaked at 12 hpi ($76.7 \times 10^5 \pm 7.65$)and showed a stark decline by 20 hpi ($16.0 \times 10^5 \pm 4.76$), returning to baseline conditions by 24 hpi. In T_S fish, neutrophil recruitment was at a significantly lower level from 4-12 hpi, with the highest infiltration at 16 hpi ($72.4\times 10^5 \pm 10.5$) compared to T_D fish. By 24 hpi T_S fish neutrophils had not returned to baseline levels. Although total leukocyte number was not statistically different at 8 hpi in both temperature conditions, the proportion of neutrophils from that total leukocyte population was statistically significant. Macrophage/monocyte recruitment peaked at 16 hpi in both temperature conditions, but macrophage/monocyte count had not returned to baseline by 24 hpi in T_S fish. TD fish macrophage/monocyte counts dropped significantly at 20 hpi ($5.05 \times 10^5 \pm 3.10, p < 0.05$)compared to T_S fish ($21.0 \times 10^5 \pm 3.10, p < 0.05$). The lymphocyte population made up the smallest proportion of total leukocytes. At multiple time points within 24 hours, the lymphocyte population of T_D fish was higher than T_S fish (t=4 hpi, 12 hpi, 20 hpi, 24 hpi). Lymphocytes gradually increased in T_D fish, showing highest count at 24 hpi ($25.3 \times 10^5 \pm 3.35$) while in T_S fish, lymphocytes remained consistently below $2x10^6$ and at 24hpi ($4.55 \times 10^5 \pm 3.35$) were like the 4 hpi numbers. While behavioral fever altered and accelerated leukocyte recruitment kinetics, for many timepoints (t= 0 hpi, 4 hpi, 8 hpi, and 16 hpi) the cell populations remained similar. Overall, in T_D fish, phagocyte subsets, namely neutrophils and macrophage/monocytes showed a rapid influx and an earlier egress from the peritoneum compared to T_S fish. Furthermore, lymphocytes showed a consistent increase in dynamic conditions compared to static conditions. These results suggest that behavioral fever promotes a rapid infiltration of phagocytic cells followed by a shift to the promotion of inflammation resolution.

While the febrile response altered both infiltration and composition of the leukocyte subset, to assess whether there were functional differences from fever, I examined the antimicrobial responses executed by the leukocytes.

Interestingly, the amount of ROS-positive (referred to as ROS+) cells were greater overall in fish held at static temperatures compared to fish allowed to exhibit a natural behavioral fever (**Figure 3.2.3A**) The highest levels of ROS+ cells were seen at 16 hpi in both temperature conditions however, the T_S fish had significantly higher numbers of ROS producing cells($T_S = 1.33 \times 10^5 \pm 9.31, T_D = 15.9 \pm 9.31, p < 0.05$). This data suggests that while T_D fish had a faster leukocyte infiltration post immune challenge, which was predominantly a neutrophil population, the ability to behaviorally thermoregulate does not induce an increase in their antimicrobial activity.

When I examined the NO producing cells in both temperature conditions, T_D fish exhibited significantly higher levels of NO-positive (NO+) cells compared to T_S fish at 12 hpi ($T_S = 6.83 \times 10^5 \pm 6.39, T_D = 18.8 \pm 6.39, p < 0.05$)and 16 hpi $T_S = 5.23 \times 10^5 \pm 3.02, T_D = 30.5 \pm 3.02, p < 0.05$) (**Figure 3.2.3B**). Furthermore, T_D fish were able to resolve inflammation more efficiently, as demonstrated by the sharp decline of NO+ cells as early as 20 hpi. Comparatively, NO+ cells in T_S fish did not return to 0 hpi conditions even up to 48 hpi. Taken together, while the ability to exhibit a natural fever alters leukocyte recruitment kinetics, behavioral thermoregulation changes the antimicrobial responses utilized by leukocytes in pathogen clearance.

3.3 Identification of thrombocytes using cytochemical staining, flow cytometry, and gene expression

3.3.1 Cytochemical Staining of Thrombocytes

The morphology of thrombocytes changes upon activation in both humans (platelets) and teleost (Gear 1984; Hasegawa et al. 1998). Moreover, the nucleated morphology of thrombocytes and erythrocytes add difficulty in enumeration if standard Wright-Giemsa cytochemical stains are used. In other teleost models, the Periodic Acid Schiff (PAS) stain was utilized to identify

thrombocytes in peripheral blood cultures (Ksenija Aksentijević et al. 2023). The PAS stain allowed for the distinguishing of thrombocytes from other cell types due to a high glycogen content in thrombocytes compared to other cell types. Therefore, to identify thrombocytes within the peritoneum during acute inflammation, I utilized the PAS staining approach.

While using Wright-Giemsa nuclear stains for neutrophils, macrophages, monocytes, and lymphocytes allows for the identification of the different cell types, **Figure 3.3.1A** highlights the visible similarities between thrombocytes and erythrocytes in goldfish. However, due to distinct differences in glycogen content of thrombocytes and erythrocytes, thrombocytes appear with a dark pink and purple cytoplasm while erythrocyte cytoplasm remains unstained (**Figure 3.3.1A**). After validating the distinguishing capacity of the PAS stain, I enumerated the thrombocytes within the peritoneum in both dynamic and static conditions.

While the number of thrombocytes was significantly lower than other cell populations, like my previous findings, thrombocyte recruitment was altered when fish were allowed to exhibit a febrile response. In T_D fish, there was an early increase of thrombocytes at 8 hpi which subsequently returned to baseline levels at 24 hpi (**Figure 3.3.1B**). While a slight increase in thrombocytes is observed at 16 hpi, it is not statistically significant. In T_S fish, thrombocytes were significantly upregulated at 8 hpi, 16 hpi and 20 hpi. Further, between both static and dynamic conditions, thrombocyte populations were significantly different at 12 hpi($T_S = 2.99 \times 10^5 \pm 0.191, T_D = 1.37 \pm 0.191, p < 0.05$) and 20 hpi($T_S = 9.66 \times 10^5 \pm 1.22, T_D = 0.978 \pm 1.22, p < 0.05$). To ensure that the thrombocyte infiltration was not in response to tissue injury from injection requiring thrombocytic hemostasis, I injected additional fish with 1X PBS $^{-/-}$ and enumerated any cells that infiltrated the peritoneum. In all controls, there were no thrombocytes detected after injection with 1X PBS $^{-/-}$.

3.3.2 Goldfish thrombocytes can be detected using imaging cytometry after staining with a monoclonal antibody.

Thrombocytes and thromboid lineage cells contain a G6F-like transmembrane molecule (Ohashi et al. 2010) which can be detected via monoclonal antibodies. I utilized the 5H2 monoclonal antibody that binds to G6F-like transmembrane domains found on thrombocytes and occasionally erythrocytes. After optimizing the antibody assay, I was able to capture images of

thrombocytes isolated from the peritoneal cavity of goldfish during acute inflammation (**Figure 3.3.2**). However, the authors that designed this hybridoma cell line mentioned occasional binding to erythrocytes. To ensure that this antibody stained only thrombocytes, I collected blood from these goldfish and clotted it. Assuming most, if not all thrombocytes participated in the clot, I collected the remaining non-coagulated blood fraction and stained it using this antibody. Additionally, I stained an aliquot of this non-coagulated blood fraction with both Wright-Giemsa and PAS stains, to confirm that this fraction contained only erythrocytes. The cytochemical stains confirmed that there were no thrombocytes in the blood fraction and when stained with the monoclonal antibody, there was less than 1% binding. Therefore, **Figure 3.3.2** includes three representative cells that are positive (indicated by POS) for the primary monoclonal antibody and secondary PE antibody, presumed to be thrombocytes and one erythrocyte from the coagulated blood fraction, negative for the primary monoclonal antibody and secondary PE antibody (indicated by NEG).

3.3.3 Alterations in peritoneal thrombocytes are reflected in the hematopoietic compartment by molecular quantification of *c-mpl.*

While *c-mpl* has been identified as a critical marker for thrombocyte proliferation and activation, I was curious to determine if it was primarily a marker for proliferation or activation (Hitchcock et al. 2021). In thrombocytes, the *c-mpl* gene encodes for the thrombopoietin receptor located on the surface of thrombocytes (**Figure 3.3.3A**). Thrombocytes originate from a myeloid early progenitor, housed in the hematopoietic compartment of goldfish, the kidneys (Katzenback et al. 2016). In the kidneys, an upregulation of *c-mpl* is indicative of an increase in commitment to differentiate into thrombocytes rather than erythrocytes. To determine whether there was greater proliferation of thrombocytes in the kidneys, I quantified *c-mpl* in the kidneys before (12 hpi), during (16 hpi) and after (20 hpi, 24 hpi) the peak of infiltration.

Overall, in T_S fish, there is a greater number of thrombocytes in the peritoneal cavity (**Figure 3.3.1B**). In the kidneys of these T_S fish, there is also a greater upregulation of *c-mpl* compared to kidneys of T_D fish (**Figure 3.3.3)**. The first significant upregulation of *c-mpl* occurs at 12 hpi, while the second and greatest upregulation of *c-mpl* occurs at 20 hpi in T_S fish. At 12 hpi $T_S = 3.99 \times 10^5 \pm 0.447, T_D = 1.76 \pm 0.447, p < 0.05$) and 20 hpi $T_S = 12.2 \times 10^5 \pm$

$1.49, T_D = 1.67 \pm 1.49, p < 0.01$) within the peritoneum there is a significantly higher thrombocyte population (**Figure 3.3.1B**) indicating that the there may be an increase in commitment to thrombocyte differentiation in the hematopoietic compartment, and these differentiated thrombocytes are subsequently recruited to the peritoneum to participate in acute inflammation. Interestingly, there is only minor upregulation of *c-mpl* in the kidneys overall, however this small upregulation results in the influx of up to 1 million thrombocytes in the peritoneum.

In contrast to static conditions, when a fish is allowed to exhibit a natural febrile response, there is no significant upregulation of *c-mpl*. However, it is worth noting that the maximum thrombocytes in the peritoneum were outside of the peak cell infiltration window (i.e., 12 hpi to 20 hpi). Therefore, there may be early upregulation of thrombocytes prior to peak infiltration window when the host is inducing inflammatory pathways rather than during the peak of inflammation. While not statistically significant from baseline, it is important to note that in T_S fish, *c-mpl* upregulation is not fully attenuated by 24 hpi and there is a sustained upregulation of *c-mpl* in the kidneys up to 24hpi ($T_S = 6.33 \times 10^5 \pm 2.06,\ p = 0.357$).

3.4 Discussion

To understand the impact of thermoregulation in ectotherms and the potential advantages of a febrile response, my first aim was to characterize total leukocyte populations within the acute inflammatory timeline in both dynamic and static temperature conditions.

In quantifying total leukocytes in dynamic and static temperature conditions, I found that there is an earlier leukocyte infiltration in dynamic temperature conditions compared to static conditions. In dynamic conditions there was significant leukocyte infiltration as early as 8 hpi compared to baseline 0 hpi, and significantly higher leukocyte infiltration by 12 hpi in dynamic conditions compared to static. The peak of infiltration was observed to be at 16 hpi in both static and dynamic conditions post zymosan injection, which differs from the 18 hpi peak reported by Havixbeck et al (2015). Furthermore, there was rapid egress of the infiltrated leukocytes by 20 hpi in dynamic conditions compared to static, and within static conditions the leukocytes remained within the peritoneal cavity for a longer period. Further, the rapid egress of leukocytes was observed in dynamic conditions as early as 20 hpi. While similar data was obtained by Wong

(2017), I did not observe the same degree of decrease in leukocyte count at 20 hpi. However, both my data and previously acquired data indicated a significant drop in leukocyte counts at 20 hpi in dynamic conditions compared to static. In simply examining leukocyte recruitment, the differences caused by allowing a natural febrile response versus keeping goldfish at static temperatures, the role of thermoregulation and temperature is highlighted.

However, the infiltrating leukocytes are a heterogenous population of innate and adaptive immune cells that participate in effector functions for both the onset and resolution of inflammation. This can include a vast array of responses such as phagocytosis, antimicrobial responses (ROS and NO production) and subsequent release of anti-inflammatory cytokines for the resolution of inflammation. To delineate the significance of thermoregulation, my second aim was to identify the subpopulations of infiltrating leukocytes. Like the reports of Havixbeck et al (2015), the total leukocyte population in static conditions predominantly consisted of neutrophils, followed by macrophages and monocytes. I found that these proportions of cellular subpopulations were accurate for both static and dynamic conditions. Specifically, as reported by previous students from my lab, there is an earlier infiltration of neutrophils and macrophage/monocytes within dynamic conditions compared to static conditions (Havixbeck et al 2015; Wong 2017). Lymphocyte recruitment to the peritoneum is greater overall in dynamic conditions and increases beyond 24 hpi.

The earlier recruitment of neutrophils and macrophages in warmer temperatures has been observed in many ectothermic fever models including lizards, Atlantic salmon, and goldfish (Pettersen et al. 2005; Bernheim et al. 1978; Havixbeck et al 2015). In mammals, several studies have shown that the elevation of body temperature results in increased neutrophil populations through the increased temperature-dependent release of G-CSF (granulocyte colony stimulating factor; Ellis et al. 2005; Capitano et al. 2012). In mice, hyperthermia also increased G-CSF concentrations, thereby increasing accumulation of neutrophils resulting in significant tumor reduction mediated by neutrophil generated reactive oxygen species (Ellis et al. 2005). In comparison, my results show that fish in febrile conditions generating lower amounts of ROS compared to afebrile (static) conditions post injection with zymosan. Alternatively, there was significantly greater amounts of NO positive cells in febrile conditions. A potential explanation for the attenuated ROS response in T_D fish could be that the greater efficiency in recruitment

kinetics of neutrophils and macrophage/monocyte populations allowed for overall increase in other pathogen clearing strategies such as phagocytosis and NETosis by neutrophils (Monteith et al. 2021). While there were fewer ROS producing cells in T_D fish, T_D fish showed faster pathogen clearance suggesting an alternative antimicrobial killing mechanism. Similar results in goldfish were observed by Wong (2017) in response to zymosan induced peritonitis and Haddad et al (2023) in dynamic conditions in response to furuncle infection with *A. veronii.* Overall, these data suggest that febrile conditions alter anti-microbial responses of leukocytes and drive these anti-microbial responses towards NO mediated killing.

Despite that many pathogenic bacteria have optimal growth temperatures in the febrile range of mammalian hosts, studies show increased amounts of pro-inflammatory cytokines released by macrophages and neutrophils including TNFα, IL-6 and IFNγ in the peritoneum of febrile hosts compared to afebrile ones (Jiang et al. 2000). Brief heat-treatment of mice prior to endotoxin exposure resulted in stronger upregulation of TNFα by macrophages and increased pro-inflammatory macrophages up to 48 hours post exposure compared to non-heat-treated controls (Lee et al. 2012). Furthermore, macrophages of heat-treated mice released significantly greater amounts of TNFα, IL1β, IFNγ and IL-6 after restimulation with endotoxin, mounting a considerably greater pro-inflammatory response compared to non-heat-treated controls (Lee et al. 2012). Mammalian studies have shown that increased temperatures have allowed for an enhancement of lymphocyte trafficking within the endothelial venules of lymph nodes. For example, heat treatment allowed for increased L-selectin-dependent adherence of naïve T lymphocytes, increased intracellular density of ICAM-1, and increased number and duration of T cell-antigen presenting cell (APC) interactions (Evans et al. 2015). These studies indicate that fever and external heat can contribute to increased pro-inflammatory responses, and overall better pathogen clearance.

Despite the benefits of mounting a stronger pro-inflammatory response and enhanced pathogen clearance, increased temperatures may also result in increased tissue injury and lead to potential pathophysiology related to chronic inflammation. Febrile-range hyperthermia accelerated pathogen clearance in murine models infected with *Klebsiella pneumoniae* but increased mouse mortality due to significant vascular endothelial injury within pulmonary tissues (Rice et al. 2005). The damage in pulmonary tissues was attributed to increased ROS release by

the neutrophil population resulting in tissue damage. However, studies have shown that a downregulation of ROS and increase in NO mediated anti-microbial killing enhanced post infection outcomes against opportunistic pathogens (Li et al. 2015). My data and data obtained by others in the lab indicated an upregulation of NO rather than ROS in febrile conditions in two infection models (Wong 2017; Haddad et al. 2023). Therefore, fever may favor alternate antimicrobial responses, thereby reducing the damaging effects of unhindered ROS-mediated pathogen clearance.

Increases in high-mobility group box 1 protein (HMGB1) often released by macrophages in inflammatory sites can exacerbate inflammatory pathology in hosts. *In vitro* studies found that HMGB1 release is attenuated at higher temperatures resulting in decreased release of pro-inflammatory cytokines in RAW264.7 macrophages (Hagiwara et al. 2007). This was partly attributed to heat treatment resulting in increased macrophage intracellular heat shock proteins (HSP), which attenuated pro-inflammatory responses by macrophages (Hagiwara et al. 2007). Heat treatment in rheumatoid arthritis murine models showed the induction of HSP70 and subsequent decrease in pro-inflammatory macrophage recruitment and function in chronically inflamed sites (Lee et al. 2015). These studies indicated that heat may provide protective benefits in innate immune pathogen clearance, however, the inability of an organism to attenuate the pro-inflammatory response and promote the resolution of inflammation can result in long term pathologies. The administration of heat may also result in a greater capacity for resolving inflammation and an efficient switch between pro-inflammatory to pro-resolving agendas within organisms. In goldfish, after infection with *Aeromonas veronii*, fish in dynamic (febrile) conditions were able to mount a greater hypothalamic upregulation of HSP70 and HSP90 compared to fish held at constant 16°C temperatures. Furthermore, like mammalian studies, febrile condition fish had significantly greater hypothalamic upregulation of IL1β, TNFα, and IL-6 earlier on in the acute inflammatory response compared to in afebrile (static 16°C) conditions (Haddad et al. 2023). The enhanced pathogen clearance and acute inflammatory outcomes may be due to fever altering the kinetics of recruited leukocytes and their functions. These alterations are seen in the significant differences in leukocyte recruitment in my data and previously acquired results from my lab (Havixbeck et al 2015; Wong 2017).

As fever plays an important role in altering the recruitment and function of leukocytes, I was curious to determine the impact of fever on thrombocytes. Thrombocytes are often referred to as the evolutionary ancestor of platelets. Platelets have been characterized to have several functions beyond hemostasis, including recruitment of leukocytes, release of pro-inflammatory and anti-inflammatory cytokines, modulating antigen presentation, and enhancing adaptive immune responses. As platelets play a diverse role in inflammation, I was curious to examine the effects of thermoregulation on thrombocyte populations. However, while there are studies that have identified thrombocytes in teleost, techniques for the isolation, and quantification of primary thrombocytes from the site of immune challenge are limited. To date, isolation methods included cytochemical stains, specific binding with monoclonal antibodies to detect via flow cytometry, and molecular quantification of thrombocyte specific receptors via qPCR.

To identify thrombocytes within the peritoneal cavity of goldfish, I utilized a monoclonal antibody that bound to the G6F-like transmembrane domain of thrombocytes (Ohashi et al. 2010). This antibody was previously reported to partially bind to erythrocytes and therefore, one of my first steps involved stimulating thrombus formation in whole blood and testing the un-clotted fraction for antibody binding. Using imaging flow-cytometry I was able to determine less than 1% binding of the mAb to erythrocytes. There have been several reports of thrombocytes in teleost having phagocytic capacities along with other immune activities. With the ability to detect thrombocytes using the imaging flow cytometer, I was curious to see whether thrombocytes contributed to the antimicrobial ROS and NO responses seen before and after acute inflammation. However, due to the limited quantity of thrombocytes within the peritoneum, after sorting the thrombocytes using a Cell Sorter and gating for cells positive for antibody binding, there were not enough thrombocytes in the fraction to preform and execute the ROS and NO assays.

The PAS stain takes advantage of the high glycogen content in thrombocytes and not erythrocytes and is therefore an important tool for distinguishing populations of erythrocytes from thrombocytes (Ksenija Aksentijević et al. 2023). Using the PAS stain I was able to enumerate thrombocytes during the window of acute inflammation. In febrile conditions the highest number of thrombocytes were present at 8 hpi, however they were not significantly different from fish held at static temperatures. Thrombocyte count remained low in febrile

conditions while at 20 hpi in static conditions, thrombocytes were significantly greater than dynamic conditions. Studies on human platelets report morphological changes in platelets in response to storage temperature changes. Storage of platelets at room temperature (20℃) resulted in increased platelet activation whereas mild heat shock of platelets at 42℃ revealed little to no changes in activation states compared to 37℃ controls (Maurer-Spurej et al. 2001). Several other differences in activation were detected in human blood platelets in response to temperature via flow cytometry including increased activation of αIIbβ3 in response to 10-minute storage at room temperature (Huskens et al. 2018). These findings indicate that platelets are sensitive to temperature and lower temperatures may result in increased activation and recruitment of platelets. These studies concur with my data as fish allowed to exhibit a natural febrile response (dynamic) showed overall lower numbers of thrombocytes compared to fish held at constant 16℃ temperatures (static). Furthermore, activation and proliferation of thrombocytes at the hematopoietic site, measured via *c-mpl* reflects thrombocytes in the peritoneum and further suggests that afebrile (static) conditions result in increased thrombocyte activation. The hypothermic sensitivity of platelets may be a conserved feature from ancestral thrombocytes, however more studies are required to determine the conservation of cold sensitivity amongst thrombocytes and platelets.

In summary, I have determined the role of fever in acute inflammation by examining the effects of fever on classical effector cell (neutrophils, macrophages, lymphocytes) recruitment and function. Subsequently, I also showed the effect of fever on thrombocytes, the ancestors of platelets, which have significant immunomodulatory capacities. Finally, I was able to quantify thrombocytes using imaging flow cytometry, cytochemical staining, and transcriptional changes and found notable differences in febrile versus afebrile conditions.

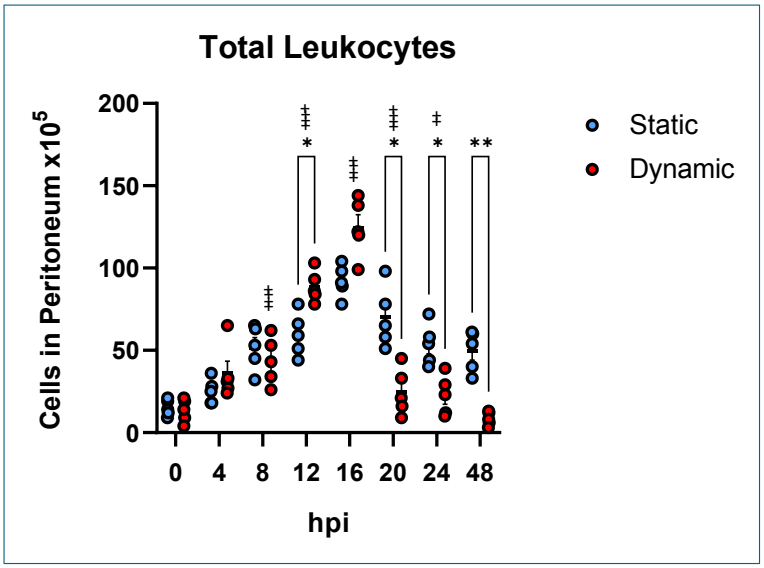
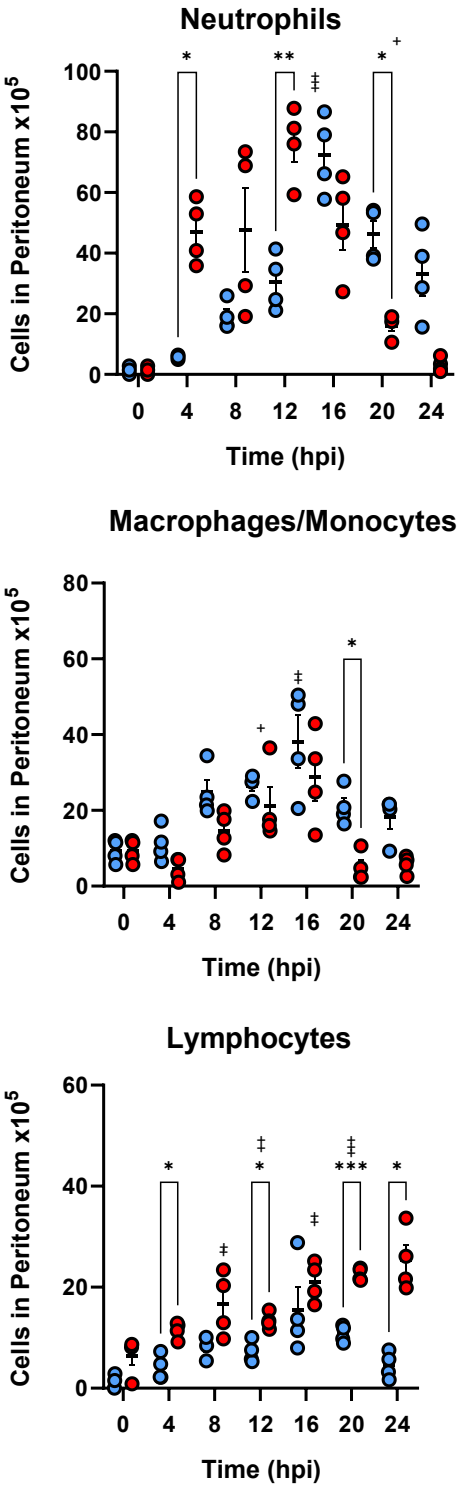


Figure 3.2.1: Total leukocyte infiltration throughout acute inflammation in static and dynamic temperature conditions.

Goldfish were challenged with an intraperitoneal injection of zymosan and housed in static 16°C water (blue) or within the dynamic ATPT (red) before isolation via peritoneal lavage and quantification via hemacytometer. The lavages were preformed over 0, 4, 8, 12, 16, 20, 24 and 48 hpi in static and dynamic temperature conditions. Each point represents one fish with a n=5 per timepoint and a total n=40 per temperature condition. Data was analysed using an ordinary one way analysis of variance (ANOVA) and Tukey's post-hoc test. Significance between groups of $p<0.05$ is denoted with (*), $p<0.001$ is denoted with (**) and significance from baseline is denoted with (+)

A


Neutrophils
Cells in Peritoneum x10^5
100
80
60
40
20
0
0 4 8 12 16 20 24
Time (hpi)
Macrophages/Monocytes
Cells in Peritoneum x10^5
80
60
40
20
0
0 4 8 12 16 20 24
Time (hpi)
Lymphocytes
Cells in Peritoneum x10^5
60
40
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Time (hpi)

B

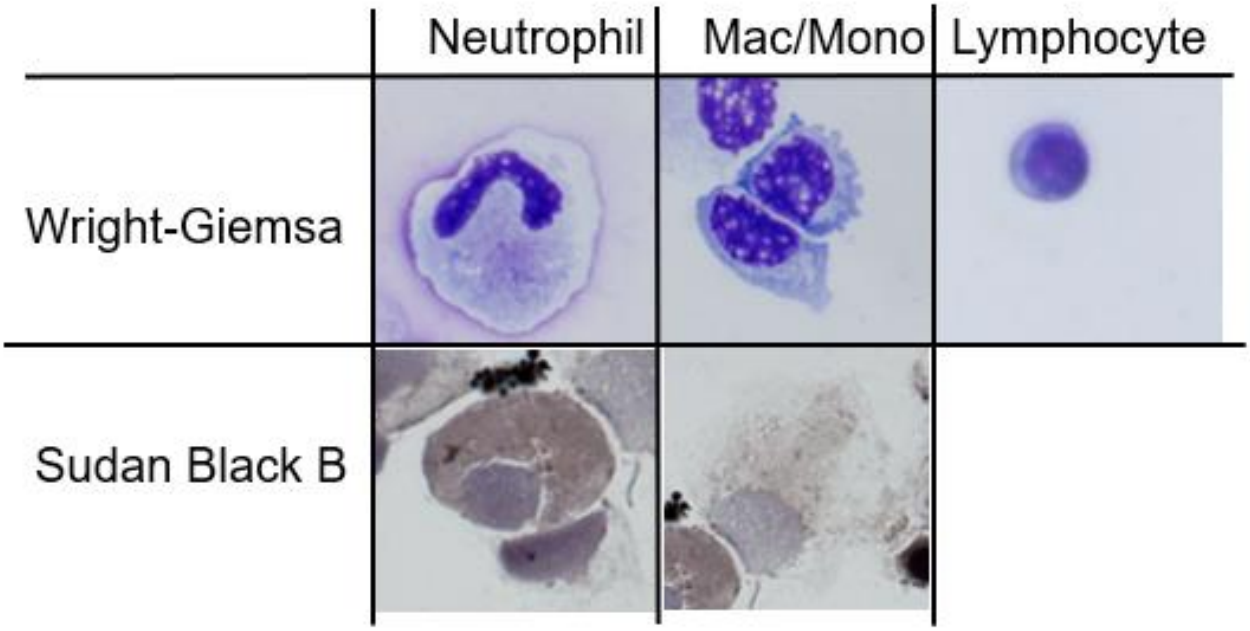


Figure 3.2.2 Behavioral fever altered recruitment kinetics of specific innate immune cell subpopulations.

Goldfish were challenged with an intraperitoneal zymosan injection and placed in either an ATPT (blue) or static (red) holding tanks where temperature was held constant at 16°C. **A**: At respective timepoints, total leukocytes were extracted by peritoneal lavage and subpopulations were further isolated using cytohistochemical staining. Each point represents one fish with a n=4 per timepoint and a total n=28 per temperature condition. Data was analysed using an ordinary one way analysis of variance (ANOVA) and Tukey's post-hoc test. Significance between groups of $p<0.05$ is denoted with (*), $p<0.001$ is denoted with (**) and significance from baseline is denoted with (+) **B** Cytochemical stains of macrophage/monocyte, neutrophil and lymphocyte populations. Macrophage/Monocyte populations stained negative for Sudan Black B while neutrophil populations stained positive, allowing quantification. Lymphocytes and macrophage/monocytes were distinguished by morphological differences visualized after staining with a Wright-Giemsa stain.

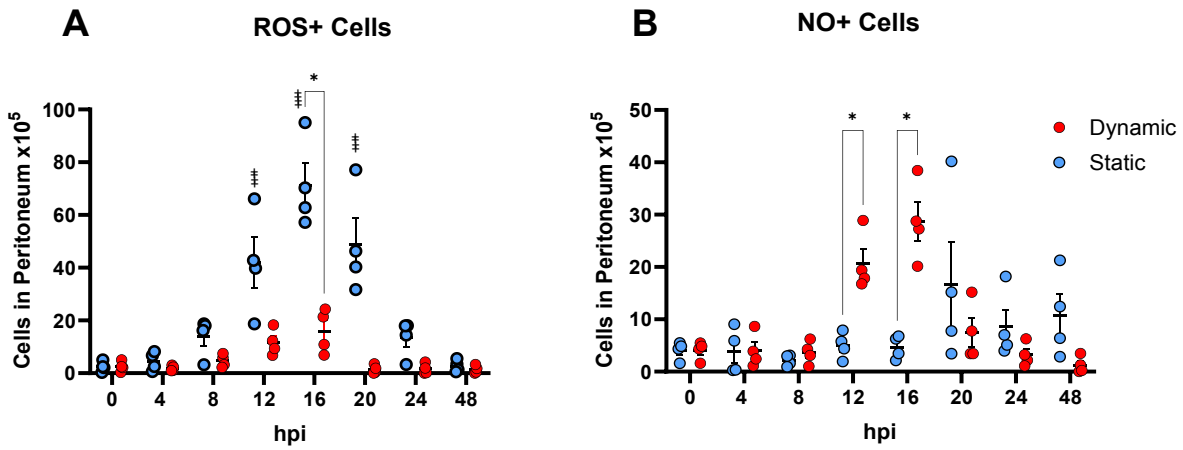


Figure 3.2.3: Behavioral fever allows for the selectivity in ROS and NO antimicrobial responses of leukocytes.

Goldfish were challenged with an intraperitoneal zymosan injection and placed in either an ATPT (blue) or static (red) holding tanks where temperature was held constant at 16°C. **A:** Cells were stained using CellRox for the detection of ROS-positive cells. **B:** Cells were stained using DAF-FM for detection of NO-positive cells. Positive cells were quantified using an Imaging Flow Cytometer. Data was analysed using an ordinary one way analysis of variance (ANOVA) and Tukey's post-hoc test. Significance between groups of $p<0.05$ is denoted with (*), $p<0.001$ is denoted with (**) and significance from baseline is denoted with (+). Each point represents one fish with a n=4 per timepoint and a total n=32 per temperature condition. Error bars represent standard error of the mean (SEM).

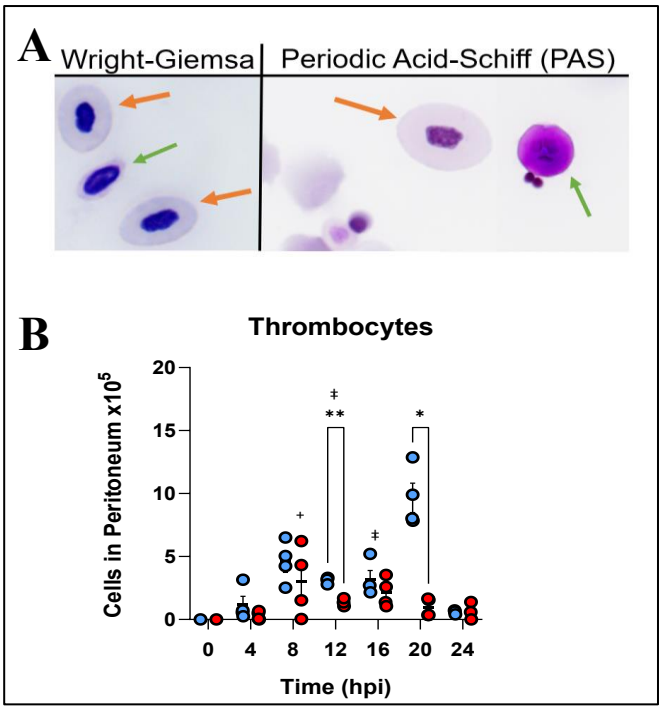


Figure 3.3.1 Behavioral thermoregulation altered recruitment kinetics of goldfish thrombocytes in-vivo.

Goldfish were challenged with an intraperitoneal zymosan injection and placed in either an ATPT (blue) or static (red) holding tanks where temperature was held constant at 16°C. **A:** Peritoneal lavages were stained with Wright-Geimsa and Periodic Acid-Schiff (PAS) cytochemical stains. Orange arrows highlight erythrocytes and green arrows highlight thrombocytes in both cytochemical stains. **B**: Thrombocytes in the peritoneum were enumerated using PAS staining in both temperature conditions across a 24-hour period post zymosan challenge. Data was analysed using an ordinary one way analysis of variance (ANOVA) and Tukey's post-hoc test. Significance between groups of $p<0.05$ is denoted with (*), $p<0.001$ is denoted with (**) and significance from baseline is denoted with (+). Each point represents one fish with a n=4 per timepoint and a total n=32 per temperature condition.

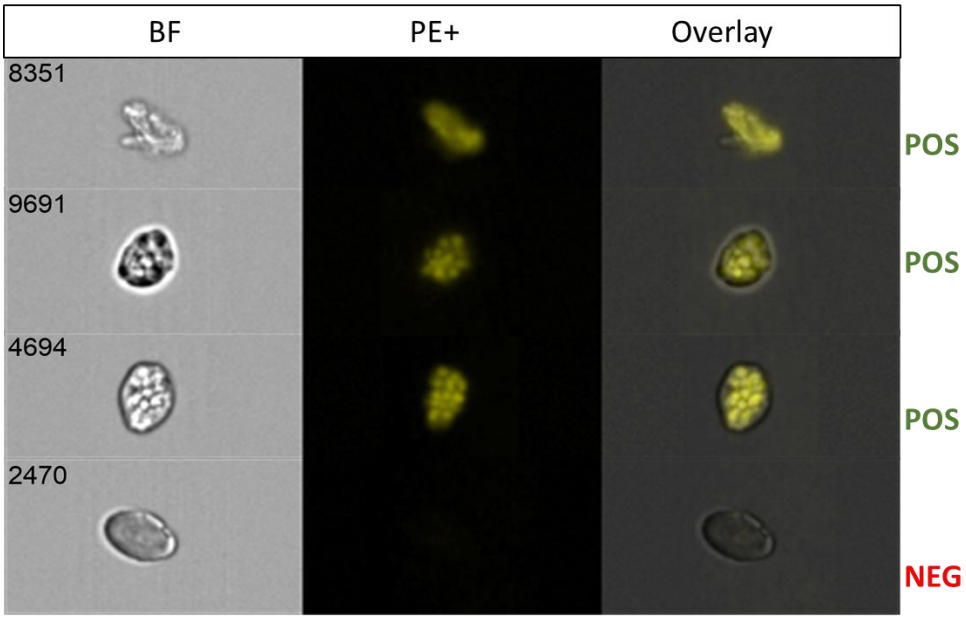


Figure 3.3.2 Specialized mucosal immunoglobulins allow for isolation and detection of thrombocytes within the peritoneum during acute inflammation.

Total blood was isolated from goldfish held at static 16℃ temperature 16 hours post injection with zymosan. Blood was coagulated via cooling and the clot was discarded. Remaining cells were stained with a primary monoclonal antibody that selectively binds to the G6F-like transmembrane domain of thrombocytes. Cells were stained with a PE secondary antibody and detected via an imaging flow cytometer. PE-positive cells are labelled POS. Erythrocytes as a morphologically similar PE-negative control are labelled as negative.

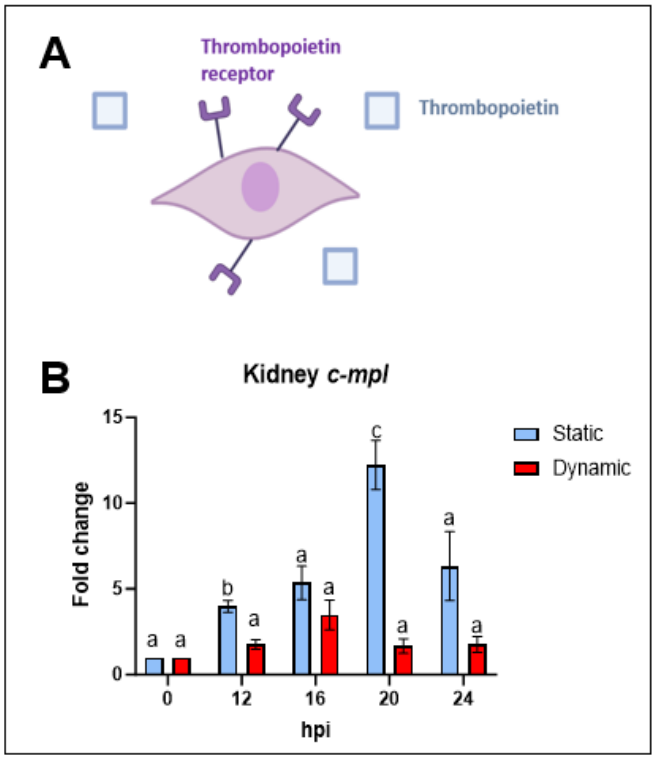


Figure 3.3.3 Molecular detection of thrombocyte proliferation in the kidney of goldfish post zymosan challenge

A: Thrombocytes proliferate through the binding of thrombopoietin (protein) to the thrombopoietin receptor, named *cmpl* found on the surface of thrombocytes. Upregulation of c-mpl in the kidneys is an indication of an increase in proliferation of thrombocytes. **B**: Goldfish were challenged with an intraperitoneal zymosan injection and placed in either an ATPT (blue) or static (red) holding tanks where temperature was held constant at 16°C. Goldfish kidneys were harvested at 0, 12, 16, 20, and 24 hours post injection with zymosan. RNA from kidneys was extracted and fold-change of c-mpl was detected via qPCR. Beta-actin was used as a reference gene with three technical replicates of 5 samples per timepoint. Data was analyzed using an ordinary two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test and alphabetical letters denote significance with at least $p<0.05$.

Chapter 4: Inhibition of fever dysregulates thrombocytes and overall tissue repair processes.

4.0 Introduction

In response to tissue injury related pain in humans, we often ingest NSAIDs. However, while NSAIDs contribute to the reduction of pain, most function as anti-pyretics within organisms (Hawkey 2005). With the advancement of pharmaceutical drug development and the allopathic medical practices, fever is often inhibited with the use of common antipyretic medication. Anti-pyretic medications most commonly are NSAIDs that have anti-inflammatory effects, thereby affecting the acute inflammatory process (Hawkey 2005). In endotherms research of the effect of anti-pyretics has been hampered by the inability in experimental settings to separate the effects of physiological stress of altering thermoregulation of the body, or by off-target side effects of systemic circulation of NSAIDs within the body (Doyle and Schortgen 2016).

The findings of others and the results of the previous chapter confirmed that thermoregulation allows for a more efficient immune response to an immune challenge in fish (Haddad et al 2023). The lack of an opportunity to exhibit a natural febrile response result in alterations of not only the immune response but impacts thrombocyte proliferation at the site of immune challenge (peritoneum) and hematopoiesis (kidney). This led me to question whether the inhibition of thermoregulation (via anti-pyretic administration) had impacts on a process that platelets and thrombocytes are canonically known to be involved in, tissue repair.

Within my lab, tissue repair experiments show greater leukocyte infiltration in *A. veronii* infected wounds of fish held at higher static temperatures (26°C) and dynamic temperatures compared to fish held at lower static temperatures (16°C) (Haddad et al. 2023). The increased early leukocyte infiltration was concomitant with greater wound closure and tissue remodelling. These enhanced tissue repair outcomes were supported by critical cytokine and HSP upregulation at higher temperatures in the hypothalamus of wounded goldfish. These findings demonstrated that temperature plays a substantial role in tissue repair processes.

Tissue repair is a complex process involving an inflammatory phase and multiple effector cells for pathogen clearance and restoration of tissue homeostasis (Soliman et al. 2021). Other studies have shown the impact of heat treatment on neutrophil, macrophage and lymphocyte

recruitment and function as discussed in the previous chapter (Evans et al. 2015). Through cytokine analysis, one can infer the involvement of innate effectors within the site of tissue damage and the role of multiple effector cells have been thoroughly outlined in mammalian studies. However, the role of thrombocytes beyond hemostasis and their involvement in tissue repair is lacking in teleost studies. Platelets are known to be an essential cell population involved in multiple phases of the tissue repair timeline, and platelet activation is a temperature sensitive process (Maurer-Spurej et al. 2001; Huskens et al. 2018). However, the role of thrombocytes in tissue repair, and the effect of fever on thrombocytes in the tissue repair process is still to be determined. Furthermore, the inhibition of a fever via NSAID administration and the consequences on tissue repair are yet to be established.

To determine the impact of fever inhibition on the fundamental process of tissue repair, I examined the impact of ketorolac (an NSAID) on tissue repair of fish wounded subcutaneously and infected with *A. veronii.* Moreover, I examined the impact of fever inhibition on thrombocytes within the tissue repair site by examining the upregulation of *c-mpl* at the site of tissue injury. This chapter focusses on the effect of thermoregulation on tissue repair through examination of tissue histopathology [1], thrombocyte involvement [2], and cytokine analysis [3] in static, dynamic and fever-inhibited conditions of fish subcutaneously infected with *A. veronii* over a 21-day period.

4.1 Inhibition of behavioral fever with ketorolac delays tissue repair

4.1.1 Histological analysis

Collagen deposition, stained as blue in **Figure 4.1**, appeared as mature stacked collagen as early as 7 dpi in dynamic conditions. Whereas disorganized collagen deposition was seen at 7 dpi in the static and ketorolac conditions. Mature stacked collagen later appears in static conditions at 14 dpi and in ketorolac conditions at 21 dpi.

Following hemostasis and acute inflammation, an integral component of tissue healing is regeneration of tissue integrity. The development of tissue integrity can be visualized via increased cell stratification. This return of tissue structure can be seen as early as 7 dpi (**Figure 4.1A)** in the dynamic condition. Comparatively, tissues in the static condition at 7 dpi are only

transitionally stratified. Further, there was no stratification seen by 7 dpi in ketorolac conditions (T_{ket}). Tissue stratification in ketorolac conditions was observed at 21 dpi (**Figure 4.1A**). Tissue integrity is complemented by the formation of the outer epidermis. Formation of a full epidermal layer is first seen in dynamic conditions at 7 dpi, in static conditions by 14 dpi and in ketorolac conditions by 21dpi (**Figure 4.1A**).

Histological analysis allowed for visualization of two particular cell types involved in tissue homeostasis, keratinocytes and adipocytes. Keratinocytes appear as early as 7dpi in dynamic conditions, visualized best at 200 μm as the small circular cells on the surface of the epidermis. Keratinocytes are sparsely seen by 21 dpi in ketorolac conditions, compared to static conditions that have a greater number on the outer epidermis (**Figure 4.1A)**. Similar patterns are seen with adipocytes for all the conditions and timepoints.

To address whether the administration of ketorolac, wounding patterns and *A. veronii* infection was causing changes in collagen deposition and maturity and tissue integrity, several control groups were utilized (**Figure 4.1B-C**). In the absence of wounding, after injection with ketorolac and exposure to our pathogen, there was no loss of collagen structure or maturity in the furuncles of goldfish (**Figure 4.1B**). In the absence of *A. veronii,* after injection with ketorolac and creation of a wound, collagen structure was initially lost due to wounding, but quickly regained by 14dpi and no lack of collagen maturation was observed (**Figure 4.1C**).

4.2 Ketorolac treatment dysregulates expression of thrombopoietin receptor in furuncle and hematopoietic site.

Upregulation of the thrombopoietin receptor (*c-mpl*) at the kidney is suggestive of thrombocyte proliferation. *c-mpl* was significantly upregulated in the kidney by 7 dpi in T_D fish ($T_D = 29.9 \pm 4.62, p < 0.05$), however the same significant upregulation is not visible in T_S and ketorolac conditions at 7 dpi (**Figure 4.2A**). An upregulation of *c-mpl* is seen in ketorolac treatment at 7 dpi compared to static conditions at 7 dpi ($T_{ket} = 10.8 \pm 4.62, T_S = 5.04 \pm 4.62$) . Significant upregulation of *c-mpl* is observed at 14 dpi in T_{ket} ($T_{ket} = 18.0 \pm 4.62$)while static and dynamic temperature conditions at 14 dpi and 21 dpi are not statistically significant from baseline (0 dpi). The combination of these results indicates an early and significant

upregulation of *c-mpl* in dynamic condition fish. This is contrary to a later upregulation of *c-mpl* when fever is inhibited through ketorolac, suggesting altered thrombocyte proliferation compared to static and dynamic conditions.

Upregulation of *c-mpl* at the site of the furuncle can be indicative of activated thrombocytes or proliferating thrombocytes. There is no significant upregulation of *c-mpl* in both static and dynamic conditions compared to Day 0 (**Figure 4.2B**). At the site of injury in T_{ket} fish, by 21dpi, there is a substantial upregulation of *c-mpl*, indicating either thrombocyte activation or proliferation as late as 21 dpi ($T_{ket} = 264 \pm 11.5, p < 0.05$). Upon examination of earlier timepoints, the difference between fever and fever-inhibited conditions appears as early as 4 dpi, with ketorolac conditions displaying a higher fold change of *c-mpl* than dynamic conditions. While dynamic conditions do not show a significant upregulation of *c-mpl* from baseline (0 dpi), at 7 dpi the dynamic conditions show a greater upregulation of *c-mpl* at the furuncle than static conditions($T_D = 29.9 \pm 9.85,\ p < 0.05$).

4.3 Treatment with ketorolac results in dysregulated cytokine gene expression

4.3.1 TNFα

Tumor necrosis factor alpha (*tnfa*) is a potent pro-inflammatory mediator. At the furuncle site, in static conditions, *tnfa* is not significantly upregulated (**Figure 4.3A**). In dynamic fever conditions *tnfa* is significantly upregulated at 14 dpi ($T_D = 12.3 \pm 2.68, p < 0.01$) compared to both 0 dpi and 7 dpi. Downregulation of *tnfa* occurs by 21 dpi in the dynamic conditions($T_D = 9.96 \pm 2.85, p < 0.01$), whereas, in ketorolac conditions *tnfa* is not upregulated until 21dpi ($T_{ket} = 15.1 \pm 2.68, p < 0.001$).

4.3.2 IL-1β

Interleukin 1-beta is conserved mediator of the inflammatory response. At the furuncle site, *il1b* shows a nonsignificant increase at 7 dpi and a return to baseline by 21 dpi (**Figure 4.3B**). A similar trend is observed in dynamic conditions. Further, in the ketorolac conditions, *il1b* is significantly upregulated at 7 dpi($T_{ket} = 40.7 \pm 9.25, p < 0.01$) and is downregulated by

14 dpi ($T_{ket} = 16.9 \pm 9.25$). Interestingly, there is a significant increase at 21 dpi for the ketorolac conditions($T_{ket} = 51.9 \pm 9.25, p < 0.0001$).

4.3.3 TGF-β

Transforming growth factor beta (*tgfb*) is a pleiotropic cytokine that can be indicative of pro- and anti-inflammatory phenotypes, dependent on the tissue. In the case of the wound site, *tgfb* is indicative of a pro-resolution agenda. In static conditions, *tgfb* is significantly upregulated 14 dpi $T_S = 150.6 \pm 25.34,\ p < 0.0001$ and 21 dpi $T_S = 107 \pm 23.9,\ p < 0.0001$ (**Figure 4.3C)**. In dynamic conditions, *tgfb* is upregulated 14 dpi $T_D = 190 \pm 23.9,\ p < 0.0001$ and 21 dpi $T_D = 153 \pm 23.9,\ p < 0.0001$, with a significantly greater fold change at 21 dpi compared to static treatment at 21 dpi. In ketorolac conditions, *tgfb* is upregulated at 7 dpi ($T_{ket} = 196 \pm 23.9,\ p < 0.0001$) to a similar degree in both static and dynamic conditions. In stark contrast, at 7 dpi in ketorolac conditions, *tgfb* is significantly greater than static and dynamic conditions at 7 dpi ($T_S = 73.7 \pm 23.9,\ T_D = 63.2 \pm 23.9,\ T_S = 196 \pm 23.9,\ p < 0.0001$). Further, by 14 dpi ($T_{ket} = 123 \pm 25.3, p < 0.001$) in the ketorolac treatment, *tgfb* is downregulated, followed by a significant increase in 21 dpi ($T_{ket} = 160 \pm 25.3,\ p < 0.0001$).

4.3.4 IL-10

Interleukin 10 (*il10*) is indicative of a pro-resolution agenda. Interestingly, *il10* at the furuncle site does not significantly increase in static or dynamic conditions (**Figure 4.3D)**. However, in ketorolac conditions, there is a significant upregulation of *il10* at day 14dpi ($T_{ket} = 11.7 \pm 2.96,\ p < 0.05$) followed by a decrease by 21dpi ($T_{ket} = 7.14 \pm 2.96,\ p = ns$).

4.4 Discussion

Histopathological analysis showed structural changes within furuncles in fever inhibited fish up to 21 dpi with *A.veronii*. Mature collagen deposition is a distinct indication of tissue healing, visualized by continuous neat stacks of collagen. The earliest appearance of mature collagen is observed in dynamic conditions when a fish is allowed to exhibit a natural fever in response to a pathogen infection. While there was collagen deposition, the maturity of the collagen in static and ketorolac treatment conditions appeared at 14 dpi and 21 dpi respectively. Collagen degradation at the time of injury leads to fibroblast proliferation and the synthesis of

growth factors involved in the proliferation and remodeling steps of tissue repair (Mathew-Steiner et al. 2021). These fibroblasts in-turn produce collagen in the latter stages of tissue remodeling and contribute to collagen maturity. The delayed appearance of mature collagen in static and ketorolac treatments may involve altered kinetics and function of fibroblasts. Pro-inflammatory cytokines have been shown to affect fibroblast migration patterns and previous studies have shown a delayed upregulation of pro-inflammatory cytokines in fixed temperature (16°C) compared to dynamic temperature conditions (Haddad et al. 2023). Furthermore, inhibition of a febrile response via ibuprofen administration in mammalian studies showed evidence of an anti-proliferative effect on fibroblasts and delayed re-epithelialization in wound healing (Landén et al. 2016). The absence of mature collagen early on in fever-inhibited conditions may be the result of a dysregulation of key pro-inflammatory cytokines which in turn affect fibroblast cell proliferation and function.

Adipocytes are present in the subcutaneous layers of mammalian and teleost skin. Classically, adipocytes have chemokine and cytokine functions in mammals, and adipocytes have been shown to impact the immune system (Zhang et al. 2023). The presence of healthy adipocytes and keratinocytes indicates properly functioning, fully remodeled tissues. The detection of adipocytes and keratinocytes by 7 dpi in febrile conditions, and the lack thereof in static and fever-inhibited conditions is a strong indicator of more efficient tissue repair in febrile conditions. Impaired wound healing occurs when an organism cannot progress through the four programmed steps in a timely manner. Inhibition of a febrile response via NSAID administration may contribute to the delay of this tightly controlled process as visualized through the lack of mature collagen and delayed detection of regulatory cells, keratinocytes, and adipocytes.

Tumor necrosis factor alpha (TNFα) is a well characterized pleiotropic proinflammatory cytokine released by macrophages and monocytes that triggers signaling cascades for other inflammatory molecules including other chemokines and cytokines. The presence of upregulated TNFα gene expression is indicative of a pro-inflammation agenda in a particular tissue (Parameswaran and Patial 2010). TNFα peaked at 14 dpi in febrile conditions and by 21 dpi returned to baseline conditions. In comparison, TNFα was significantly upregulated at 21 dpi in fever-inhibited conditions indicating that at 21dpi, the furuncle tissue is a site of pro-inflammation. TNFα has been shown to impact platelets by promoting a pro-thrombotic

effect(Pircher et al. 2012). Therefore, the upregulation of TNFα and *c-mpl* in fever-inhibited conditions at 21 dpi may be indicative of improper wound healing, resulting in a necessary upregulation of thrombocyte thrombus formation, potentially mediated by TNFα. In contrast, TNFα can stimulate platelet activation through the AA and PGE2 pathway and therefore, a dysregulation of TNFα can cause increased platelet activation at 21 dpi (Yun et al. 2016). Whether TNFα impacts thrombocytes via the AA pathway or if TNFα upregulation causes subsequent thrombocyte thrombus formation was not in the scope of this study, however high levels of TNFα are indicative of pro-inflammation present in the latter stages of wound healing, indicating that the inhibition of fever dysregulates the shutdown of pro-inflammation. Taken together with concurrent upregulation of *c-mpl*, there may be an interaction between this potent pro-inflammatory cytokine and thrombocytes.

To provide additional context about the furuncles in each treatment condition, I examined the IL1-β expression levels. IL1β is a potent proinflammatory cytokine, primarily released by activated macrophages and monocytes. Within tissue repair models, the shutdown of IL1β production is indicative of the ending of the inflammation phase (Landén et al. 2016). While in febrile conditions, IL1β gene expression peaks 7 dpi followed by a decline signaling the end of the pro-inflammation phase of tissue repair, multiple peaks are observed in fever-inhibited conditions up to 21 dpi. Multiple peaks in levels of IL1β are indicative of an active inflammatory site within those time points suggesting that fever inhibition impacts the shutdown of the pro-inflammation phase in tissue repair, potentially impacting transition to the latter stages of tissue repair. Thrombocytes may be contributing to this inflammatory site as others have demonstrated platelets participating in inflammasome activation through interactions with other leukocytes via IL1β (Kral et al. 2016). Furthermore, while there are no current investigations that indicate a thrombocyte sensitivity to IL1β in goldfish, IL1β has been characterized in multiple other teleost species and determined to have a stimulatory effect post infection (Zou and Secombes 2016).

To end the inflammation phase in tissue repair and progress further to proliferation and tissue remodeling, certain potent anti-inflammatory and pro-resolution cytokines must be upregulated. One of these includes TGFβ, a pleiotropic cytokine that antagonizes pro-inflammatory cytokines in the wound sight and promotes proliferation. Post wounding in dorsal

skin of seabream resulted in an initial increase in TGFβ at 1 dpi and 7 dpi signaling a modulatory role in inflammatory processes. While other tissue repair analysis provides excellent context on the role of key cytokines within the first week of wounding, there are no studies completed in teleost species that examine the wound site up to three weeks post infection *in vivo*. From *in vitro* studies, TGFβ has been shown to stimulate collagen synthesis, a significant component of the ECM that is necessary for tissue remodeling, in rainbow trout fibroblast cultures (Johnston 2019). Additionally, from mammalian studies, it is well established that the dysregulation of TGFβ results in tissue fibrosis due the lack of a seamless transition through each of the tissue repair phases (Duffield et al. 2013). Within a 21-day window, I observed the peak of TGFβ in both static and natural fever conditions to be at 14 dpi followed by a decline by 21 dpi. However, in the fever-inhibited conditions, we see an early peak at 7dpi followed by a decline, and subsequent increase in TGFβ gene expression. The absence of a clear upregulation and subsequent resolution indicates a dysregulation of TGFβ at the wound site. The dysregulation is paralleled with a delayed appearance of mature collagen in the histology of furuncle cross sections. Therefore, fever inhibition results in the dysregulation of a key cytokine involved in the resolution of inflammation and promotion of proliferation and tissue remodeling phases within tissue repair.

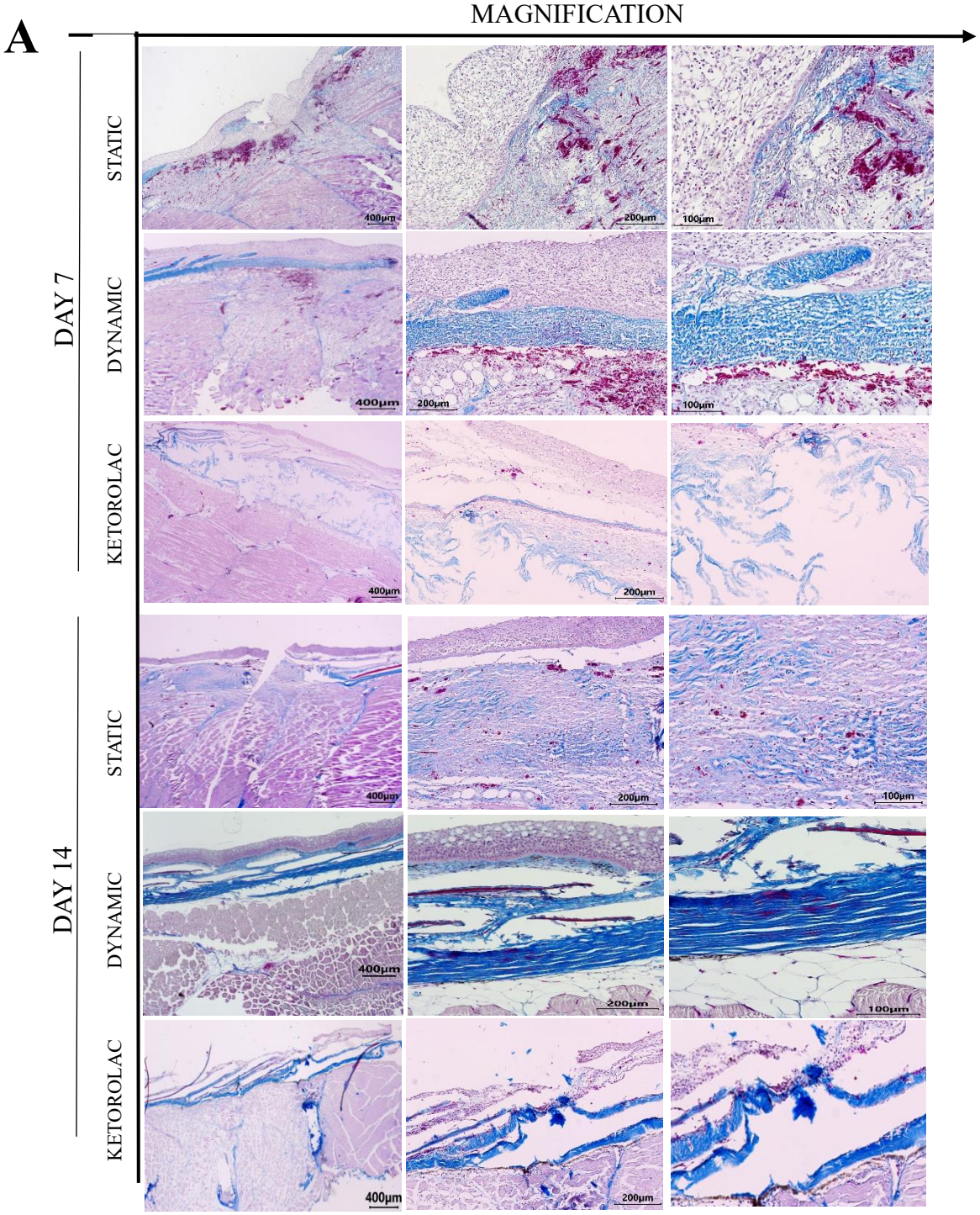
Interleukin-10 (IL10) is a cytokine highly conserved throughout evolution participating in anti-inflammatory and immunoregulatory activities. IL-10 and TNFα antagonize one another as IL-10 plays a role in stimulating B-cell differentiation and proliferation (Clarke et al. 1998). This is visible in the fever inhibited conditions where higher levels of TNFα are concurrent with lower levels of IL-10. Whether the dysregulation of TNFα leads to the dysregulation of IL-10 is currently unknown. The primary effect of IL-10 stimulation is the suppression of an immune response by professional phagocytes (Iyer and Cheng 2012). Compared to both static and natural fever conditions, fever inhibition results in an overall greater level of immunosuppression at 14 dpi. In mammals, platelets contribute to the stimulation of IL-10 production by macrophages and monocytes (Iyer and Cheng 2012). The greater proliferation of thrombocytes in the kidneys at 14 dpi may produce a greater stimulation of IL-10 production via thrombocyte stimulation of macrophages. However, thrombocyte-specific stimulation of IL-10 in teleost has not been established and remains to be further characterized.

Overall, the suppression of fever via ketorolac injection indicates a dysregulation of key pro-inflammatory and anti-inflammatory cytokines. Histopathological data shows a lack of mature collagen, key regulatory cells, and delayed cell stratification in fever inhibited conditions. This may be the product of the activity of key pro- and anti-inflammatory cytokines. Inhibition of fever alters thrombocyte proliferation and activation. Due to thrombocyte activities in tissue repair models and interactions with key innate immune cells, thrombocytes may be contributing to the delayed tissue repair and dysregulated cytokine profile in fever inhibited conditions. A 6-to-8-hour bioactivity of ketorolac in the initial stages post wounding results in significant changes in tissue repair and thrombocyte activation, further stressing the benefit of a febrile response in tightly structured processes such as tissue repair.

To determine the role of thrombocytes within the wound healing process, I utilized qPCR quantification of a prominent thrombocyte cell surface receptor, *c-mpl*. Classically, upon platelet activation in response to tissue injury, platelets influence the inflammatory process through membrane receptors and soluble mediators (Eisinger et al. 2018). The receptors and mediators are involved in the promotion of inflammation, angiogenesis, and cell-cell interactions among innate and adaptive immune cells. Therefore, I hypothesized an early thrombocyte upregulation in febrile conditions, and a delayed response in static and fever-inhibited conditions. The early upregulation of *c-mpl* in febrile conditions may be a beneficial process that aids in progression of wound healing. Within 7 dpi, the upregulation of *c-mpl* in kidneys was observed. Upregulation of *c-mpl* in the kidneys may be an indicator of an increase in thrombocyte proliferation. In static and fever-inhibited conditions, the same upregulation was not observed, therefore the lack of a natural febrile response may impact thrombocyte population and subsequent contributions to tissue repair. The consistent upregulation of *c-mpl* in the furuncle may be indicative of thrombocyte activation and contribution to each phase of wound healing. Compared to febrile conditions, which displayed a consistent upregulation up to 21 dpi, ketorolac administration resulted in a significant upregulation at 21 dpi. Taken together with tissue histology, this delayed appearance of thrombocyte activation may be the result of thrombocyte dysregulation due to ketorolac administration. NSAIDs have been shown to have an antiplatelet and anticoagulant effect where administration of NSAIDs in rats and mice has impacted the stimulation of angiogenesis by platelets(Su et al. 2010).. Furthermore, chronic usage of NSAIDs in humans has

resulted in clotting pathologies in multiple long-term studies (Zhao-Fleming et al. 2018; Haws et al. 1996).

In summary, this chapter examines the effects of fever-inhibition on restoration of tissue homeostasis, tissue repair. Multiple cell subsets and cytokines are involved within the phases of the tissue repair process and the dysregulation of the progression through phases of tissue repair results in impaired tissue repair. The inflammatory phase of tissue repair is a critical step, and the administration of NSAIDs to reduce pain and inflammation may result in improper wound healing. The data in this chapter shows delayed tissue repair up to 21 days post infection in ketorolac treated fish, compared to dynamic (T_D) fish. Via histology, significant markers of sufficient tissue remodelling (ie. collagen deposition, cell stratification, adipocyte and keratinocyte proliferation) appear days later in the fever inhibited (T_{ket}) fish compared to T_D fish. Furthermore, a single dose of ketorolac results in the dysregulation of key pro-and anti-inflammatory cytokines. Within these tissue repair processes, there is delayed thrombocyte gene *c-mpl* upregulation in fever-inhibited conditions and greater thrombocyte proliferation at the sites of hematopoiesis suggesting that fever-inhibition may hyperactivate thrombocytes in tissue repair processes. These data highlight the damaging effects of fever inhibition on tissue repair outcomes on the molecular, cellular and tissue level.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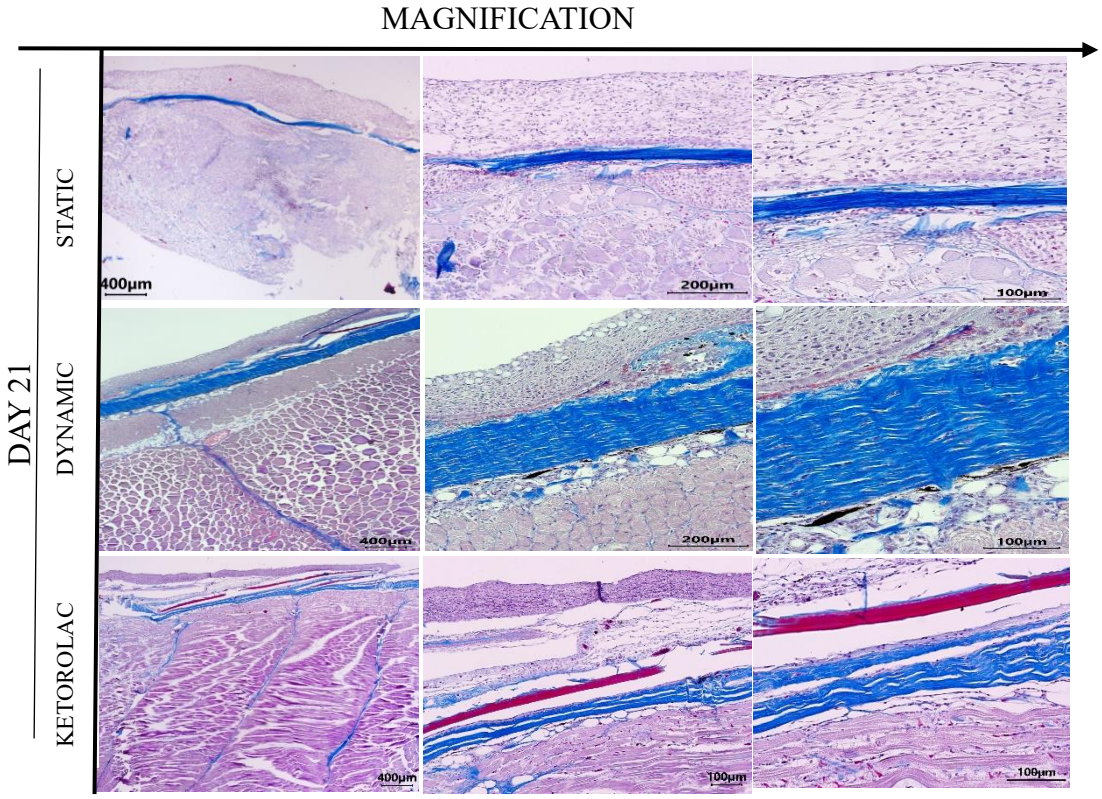
MAGNIFICATION
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B

MAGNIFICATION

(+) KETOROLAC

(-) WOUND

(+) *A. veronii*

DAY 7

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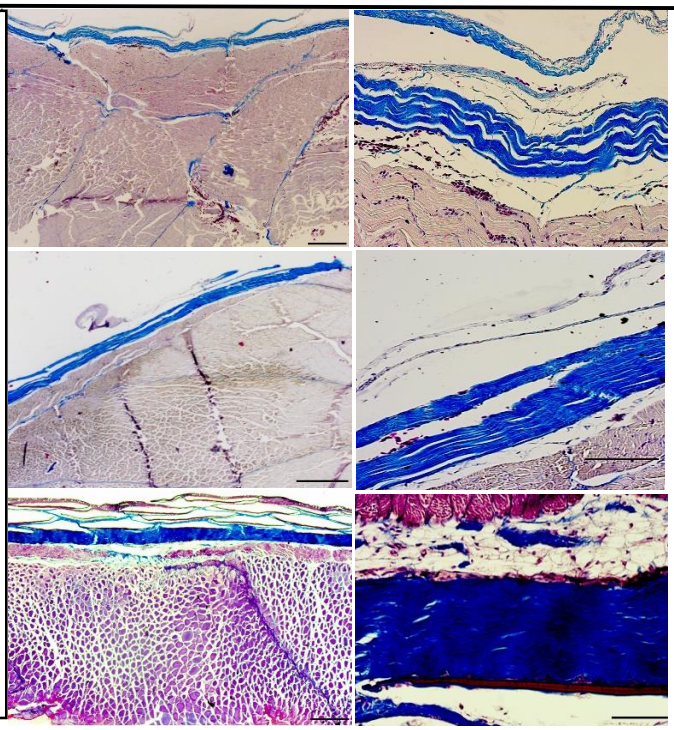
DAY 21



C

MAGNIFICATION

(+) KETOROLAC

(+) WOUND

(-) *A. Veronii*

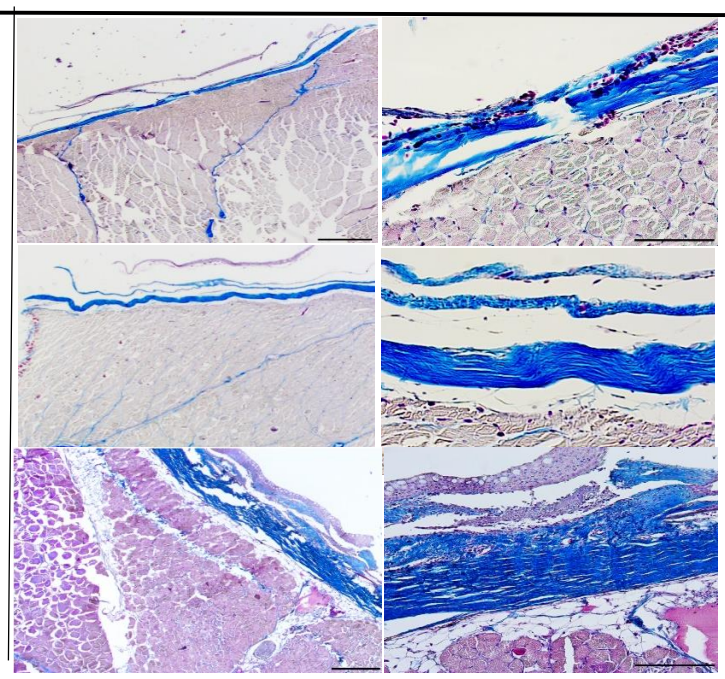


Figure 4.1 Histopathology of goldfish furuncles in static, dynamic and fever-inhibited conditions Inhibition of fever with Ketorolac causing delayed tissue reconstruction and wound healing up to 21 days post infection. **A:** Histological staining of furuncle tissue structure and collagen in three temperature conditions (Static 16℃, Dynamic, and Ketorolac-inhibited) along a 21-day infection timeline. Timepoints capture 21 days post infection with magnifications of 400µm, 200µm, and 100µm respectively. **B:** Histological staining of tissue structure and collagen in fish placed in dynamic temperature conditions after the addition of ketorolac, and *A. veronii* in the absence of a wound. Timepoints capture 7-, 14- and 21-days post infection with magnifications of 400µm and 100µm. **C**: Histological staining of tissue structure and collagen in fish placed in dynamic temperature conditions after ketorolac injection and wounding, in the absence of *A. veronii*. Timepoints capture 7-, 14- and 21-days post infection with magnifications of 400µm and 100µm.

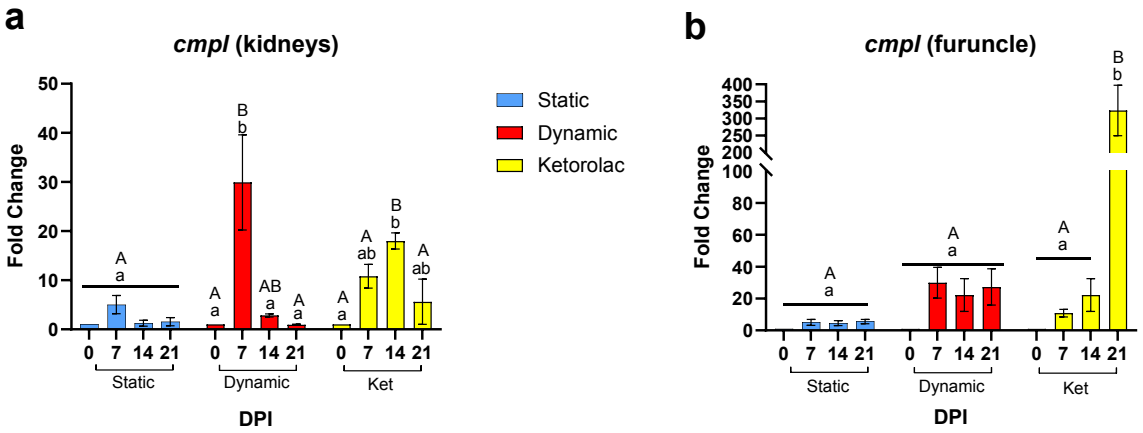


Figure 4.2: Thrombocyte proliferation and activation is dysregulated at furuncle and hematopoietic site with fever inhibition

Thrombocyte proliferation and activation measured by the thrombopoietin receptor (*cmpl*) in qPCR at the (**a**) hematopoietic and (**b**) furuncle site. Kidneys and furuncle tissue were harvested at 0, 7, 14, and 21 days post cutaneous inoculation with *Aeromonas veronii* where fish were held in three conditions: Static (16°C), Dynamic, and Ketorolac-inhibited. Beta-actin was used as a reference gene with three technical replicates of 5 samples per timepoint. Data was analyzed using an ordinary two-way analysis of variance (ANOVA) and Tukey's post-hoc test. With a minimum $p<0.05$, significance between treatments is denoted with capital alphabetical letters and differences within a treatment between timepoints is denoted with lowercase capital letters.

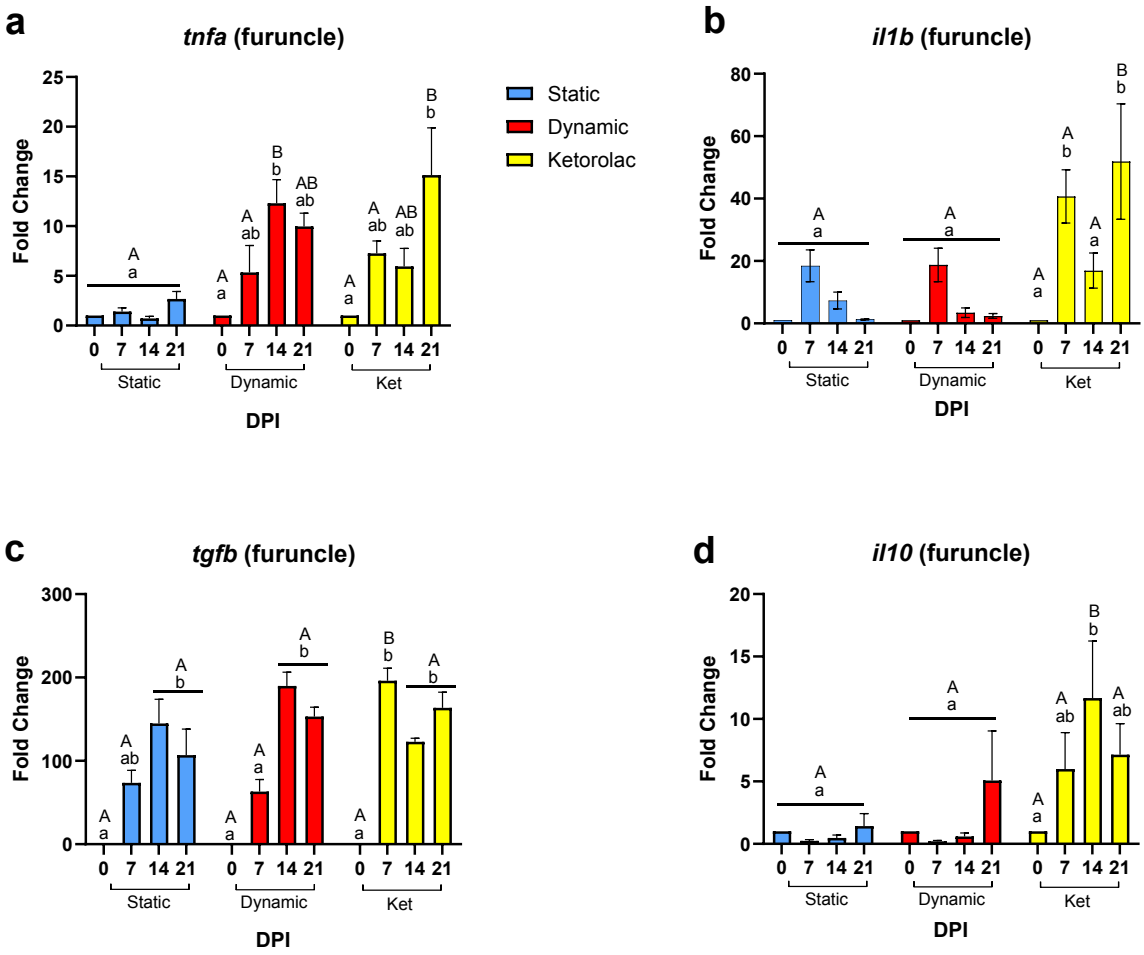


Figure 4.3 Quantitative PCR of furuncle tissue demonstrating the dysregulation of common pro- and anti-inflammatory cytokines of fish placed in three conditions: **Static (16°C), Dynamic, and Ketorolac-inhibited**.

Pro-inflammatory cytokines include (**a**) *tnfa* and (**b**) *il1b*, and anti-inflammatory cytokines include (**c**) *tgfb* and (**d**) *il10*. Fish were harvested 0, 7, 14, and 21 days post cutaneous inoculation with *Aeromonas veronii.* Beta-actin was used as a reference gene with three technical replicates of 5 samples per timepoint. Data was analyzed using an ordinary two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. With a minimum $p<0.05$, significance between treatments is denoted with capital alphabetical letters and significant differences within a treatment between timepoints is denoted with lowercase capital letters.

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

5.1 Discussion

Increased body temperature is often seen as a symptom of infection; however fever is hallmark of acute inflammation and only in the last few decades has it been proposed to be a mechanism in modulating acute inflammation (John Victor Hurley 1972). Due to fever being regarded as a symptom of infection, NSAIDs are often utilized to reduce elevated body temperature (Prajitha et al. 2019; Wrotek 2020). However, fever has been conserved for over 500 million years in evolution and therefore begs the question, is fever "good" or "bad"? Many scientists using various infection and disease models have argued for both the protective and the detrimental effects of a raised core-body temperature and therefore there is continued debate if fever has net-negative or net-positive effects.

In answering this age-old question, both endothermic and ectothermic organisms have been utilized as they both have the capacity to thermoregulate. While endothermic organisms have provided insights on the impact of temperature increase on immune cell function and activation, earlier endothermic models often utilized application of external heat to model a fever(Prajitha et al. 2019). However, application of external heat involves activation of HSPs and induction of thermal stress responses which can confound our understanding of an internally activated regulation of core body temperature (Hagiwara et al. 2007; Lee et al. 2015). Furthermore, thermal stress can alter an organism's immune response, therefore impeding our ability to delineate the effect of fever on acute inflammation. While more recent studies have attempted to model a natural fever, endothermic upregulation of PGE2 in response to pathogenic pyrogens or pyrogenic cytokines results in peripheral vasoconstriction and shivering for thermogenesis, creating variation in core body temperatures within the same cohort of organisms (Coceani et al. 1986; Evans et al. 2015). These differences can further confound the investigation of fever on acute inflammation and therefore highlight an advantage of using an ectothermic model of fever. Ectotherms contain and are sensitive to PGE2 changes within the body, but they do not possess the same capacity to metabolically increase core body temperature and thus rely on external sources of heat to raise their core body temperature (Lee et al. 2012). Ectothermic

fever models have provided incredible insights into fever mechanisms and highlighted specific contributions of fever to the innate and adaptive immune systems.

Previously in my lab, leukocytes have been shown to have pleiotropic functions. Neutrophils for example isolated before and after the peak of infiltration show pro-inflammatory and anti-inflammatory functions and regulate macrophage antimicrobial responses accordingly (Havixbeck et al. 2015). Our lab utilizes an ATPT temperature system to allow a goldfish to exhibit a natural thermal preference after immune challenge. Using this, our lab has shown that temperature alters the kinetics of leukocyte infiltration (Wong 2017). With the use of cutaneous wounds infected with *A. veronii,* leukocyte infiltration in the furuncles was greater at higher static temperatures and dynamic temperature conditions compared to colder static temperatures (Haddad et al. 2023). In response to fungal mimic injections, previous students noted key differences in leukocyte subpopulations when a fish is allowed to exhibit a natural fever response compared to static temperature conditions (Wong 2017). These marked differences in leukocyte infiltration kinetics also impact overall antimicrobial responses of the total leukocyte population. Taken together, these studies have highlighted the effects of fever, and demonstrated an enhanced immune response in febrile fish. Therefore, the aim of my thesis research was to examine the impact of fever on cell populations previously studied in the lab including neutrophils, macrophage/monocytes and lymphocytes, along with examining a new population, thrombocytes.

Thrombocytes are often referred to as the ancestors of platelets and the functional equivalent of mammalian platelets (Nagasawa et al. 2014). While mammalian platelets are well studied, knowledge of teleost thrombocytes is still emerging. Thrombocytes have been identified in many fish, amphibian, and avian species and show a remarkable degree of similarity in morphology amongst these species (Jagadeeswaran et al. 1999; Ferdous and Scott 2023; Ferdous 2014; Stosik et al. 2019). Significant morphological differences are present in thrombocytes and platelets, a major difference being thrombocytes contain a nucleus while mammalian platelets are anucleate. Other similarities and differences continue to be highlighted but it is becoming increasingly evident that platelets and thrombocytes serve many important functions beyond hemostasis.

Thrombocytes, like platelets, have the molecular machinery to participate in pathogen capture, antigen presentation, and other immunomodulatory functions via cytokine production (Table 1.6). Further, platelets are known to be sensitive to temperature changes (Huskens et al. 2018). Therefore, this understudied cell type may provide additional context on the effect of thermoregulation in acute inflammation. Thus, my project involved the quantification of various cell subpopulations and their functions, in dynamic and static conditions in response to immune challenge. In this project, I quantified thrombocytes using multiple parameters, including cytochemical staining, flow cytometry and molecular qPCR quantification. Building on previous studies from my lab, I noticed several differences in cell subpopulations including the early infiltration of professional phagocytes (neutrophils and macrophage/monocytes) along with greater engagement of adaptive leukocytes (lymphocytes) in dynamic temperature conditions. Furthermore, there was a preferential upregulation of NO-mediated antimicrobial killing in dynamic conditions compared to the ROS dominated antimicrobial response in static conditions. Thrombocytes were higher in count in static conditions compared to dynamic conditions, and thrombocytes increased at earlier timepoints in dynamic conditions, but did not significantly peak as static conditions. These trends were also observed at the level of proliferation at the site of hematopoiesis (kidneys), with T_S fish displaying higher fold-change in *c-mpl*, the thrombopoietin receptor, a marker for thrombocyte proliferation and activation. The higher levels of *c-mpl* observed in T_S fish may be due to cold sensitivity of thrombocytes, a property that has been previously identified in platelets, in which cooler temperatures have an activating effect on platelets. Alternatively, thrombocytes may have increased for a brief period in T_D fish between measurement points (between 4-8 hpi) and egressed without being captured. Thrombocytes have also been previously shown to have phagocytic capacities and engulf smaller targets than other leukocytes within blood fractions (Nagasawa et al. 2014). Therefore, another explanation necessitating higher numbers of thrombocytes within the peritoneum could be to clear up remaining pathogens or debris in T_S fish and are not required in T_D fish as T_D fish show an overall faster and efficient progression through acute inflammation. While the reason behind higher numbers of thrombocytes was not within the scope of my project, marked differences between thrombocytes in response to temperature in the site of immune challenge was observed. Taking the context of other leukocyte populations and fever enhancing the immune response in

goldfish, the presence of thrombocytes in the later stages of the acute inflammatory window (>20 hpi) may be indicative of unresolved inflammation.

To further examine the impact of fever on acute inflammatory outcomes, I utilized a well-established subcutaneous tissue repair model optimized by previous lab members (Soliman et al. 2021; Haddad et al. 2023) . The aim of acute inflammation in response to infection is restoration of homeostasis (Chen et al. 2018). An ideal model to understand restoring homeostasis is the tissue repair process. Restoration of tissue homeostasis involves four key phases: hemostasis, inflammation, proliferation, and tissue remodelling (Wallace et al. 2019). These tightly regulated phases involve a myriad of innate and adaptive effector cells, cytokines, and chemokines as well as other cell populations that contribute to tissue integrity. Chronic inflammatory pathologies and improper wound healing have been attributed to an improper progression between stages of tissue repair, primarily aberrant progression through the inflammatory stage in mammalian studies(Landén et al. 2016). The tissue repair process has been highly conserved throughout evolution and requires tight regulation and coordination of many of the previously discussed components of the immune system. Teleost thrombocytes, in addition to their hemostatic capacities, have phagocytic capacities and the molecular capacity to modulate several key pathways in tissue repair(Nagasawa et al. 2014). Therefore, one of my aims was to quantify thrombocytes in the tissue repair process. Further, I examined the impact of fever inhibition through NSAID administration on tissue repair outcomes.

Fever is often regarded as a symptom rather than a tool or aspect of acute inflammation. Therefore, to mitigate cases of hyperpyrexia (uncontrolled increase in body temperature), antipyretics such as NSAIDs are used in the treatment of fevers. NSAIDs reduce inflammatory symptoms through inhibition of COX1 and/or COX2 enzymes, limiting the effects of PGE2 on various bodily tissues (Gunaydin and Bilge 2018). Molecular quantification of thrombocyte marker *c-mpl* showed significant differences between dynamic (T_D) and fever-inhibited (T_{ket}) fish. T_D fish showed higher levels of thrombocyte proliferation at 7 dpi in the kidneys while T_{ket} fish showed greater levels of thrombocyte proliferation at 14 dpi. The inhibition of fever resulted in later upregulation of *cmpl* and a significantly greater level of *cmpl* in furuncles compared to T_D fish and T_S fish. T_D fish demonstrated an upregulation of *cmpl* that was downregulated by 21 dpi while T_S fish showed lower levels of *cmpl* overall. To determine whether high levels of

thrombocyte activation and proliferation were beneficial or harmful, I examined the tissue histology of furuncles and quantified key pro-inflammatory and anti-inflammatory cytokines within furuncles in all temperature conditions over 21 days. Tissue histology showed T_D fish regaining structural components (collagen, adipocytes, keratinocytes, cell stratification) faster than T_S and T_{ket} fish. T_D fish showed complete tissue remodelling by 14 dpi while T_{ket} fish showed tissue remodelling by 21 dpi. When cytokines within the furuncle tissues were examined, T_{ket} fish displayed high levels of pro-inflammatory cytokines that did not reduce to baseline conditions by 21 days, while pro-inflammatory cytokines in T_D fish returned to baseline conditions. Anti-inflammatory cytokines rose significantly in T_D fish and T_S fish at 14 dpi and decreased by 21 dpi, while T_{ket} fish showed varying levels of anti-inflammatory cytokines at 7-21 dpi. Overall, T_{ket} fish showed dysregulation of pro- and anti-inflammatory cytokines. Coupled with the histological images of furuncles, my data showed fever inhibition resulted in poor tissue repair outcomes and altered thrombocyte proliferation and activation.

Taken together, my results show the impact of thermoregulation on key effector cells within the immune system and outlined the kinetics of thrombocytes within the site of immune challenge. Using a short zymosan induced peritonitis model, and an *A.veronii* infected wound model, my data also supports that fever enhances immune response to pathogens. Furthermore, the inhibition of fever via NSAID administration resulted in delayed tissue repair, and dysregulation of pro- and anti-inflammatory cytokines and thrombocyte proliferation and activation in wounds. Overall, my data provides another argument for the potential net-positive impact of fever in acute inflammatory processes.

5.2 Future Directions and Limitations

5.2.1 Assessing thrombocyte function in the site of immune challenge.

Using the dynamic fever model (ATPT), I was able to demonstrate a change in thrombocyte count within static and dynamic conditions after zymosan challenge. Further, thrombocytes in teleost have been shown to have potent immunomodulatory capacities and communicate with cells of the innate and adaptive immune system (Table 1.6). Additionally, temperature has been shown to have an activating effect on human blood platelets (Huskens et al. 2018;Windberger et al. 2020). Therefore, truly gain an understanding of the effects of the febrile

response on thrombocytes, further isolation and functional assays are required to determine if there are alterations to thrombocyte function within the host in response to fever. Thrombocytes have previously been examined using highly specific antibodies in flow cytometry and shown to have phagocytic capacities (Nagasawa et al. 2014; Ohashi et al. 2010). In my study, I was able to utilize a monoclonal antibody that binds to thrombocytes with high specificity. However, due to the limited number of thrombocytes, I was not able to run additional assays assessing thrombocyte function. With developed assays in my lab, future experiments assessing the ROS and NO profiles of thrombocytes in static and dynamic temperature conditions can allow for determining if thrombocytes can release reactive oxygen species and reactive nitrogen intermediates for pathogen clearance. Furthermore, thrombocytes preferentially phagocytose smaller particles compared to other leukocyte populations (Nagasawa et al. 2014). In dynamic conditions, there is an earlier leukocyte infiltration, a preferential upregulation of NO-mediating pathogen clearance, and an earlier shift to the resolution of inflammation (Wong 2017). The dynamic conditions show an overall more efficient pathogen clearance and an earlier acute inflammatory response. The earlier increase in thrombocytes in dynamic conditions followed by lower thrombocyte count may be due to the enhanced pathogen clearance by professional phagocytes in dynamic conditions. Within my study however, I was able to assess overall count and not function. This study was limited by the lack of annotated thrombocyte genes within the goldfish genome and may be better approached through the examination of functional molecular markers of thrombocytes in the future. Other teleost models such as zebrafish have a greater number of annotated thrombocyte genes that may allow for better quantification of thrombocyte function in response to static and dynamic temperature conditions (Katzenback et al. 2016).

Thrombopoietin acting on the receptor c-mpl is an indicator of platelet count as, in mammals, it has been shown to influence megakaryocyte proliferation and differentiation, which in turn results in greater platelet production (Vianello et al. 2014). In carp, thrombocytes have been identified as cells gated positive for both c-mpl and CD41, and the addition of recombinant thrombopoietin produced thrombocytic colonies suggesting that both thrombopoietin (TPO) and it's receptor c-mpl are highly conserved molecular regulators of thrombopoiesis (differentiation into thrombocytes and proliferation of thrombocytes) (Katakura et al. 2015). Many studies have indicated that quantification of c-mpl can be used to identify thrombocytes however, it remains unclear if c-mpl levels at a particular tissue indicate thrombocyte proliferation or activation.

While measurement of cell-surface c-mpl can indicate the presence of a thrombocyte, thrombocytes will upregulate c-mpl on their surface when activated and therefore the level of c-mpl in the total mRNA of a tissue may due to high numbers of thrombocytes and or high activation of thrombocytes. Within the kidney, the high levels of *cmpl* can be attributed to proliferation, as the kidney was not the site of immune challenge and is rather the site of differentiation for many cell types in goldfish, a more accurate representation of proliferating cells and activated cells can be achieved through Bromodeoxyuridine assays. Therefore, with collection of thrombocytes from both the kidneys and peritoneum, and staining with BrdU, thrombocyte proliferation and activation can be quantified at all timepoints and conditions.

5.2.2 Assessment of leukocyte function in response to fever-inhibition

In previous studies conducted examining tissue repair outcomes in response to temperature conditions, altered leukocyte infiltration was observed when fish were placed at higher static temperatures and dynamic temperatures (Haddad et al. 2023). Furthermore, the fastest tissue repair occurred when a fish was allowed to exhibit a natural febrile response. Tissue histology showed earlier cell stratification, appearance of keratinocytes and astrocytes and maturation of collagen in dynamic conditions and 26°C static conditions. These large-scale observations were concurrent with enhanced pro-inflammatory cytokine and HSP upregulation in the hypothalamus of fish. While increases in pro-inflammatory cytokines are indicative of subsequent recruitment and activation of leukocytes and allow for the initiation of antimicrobial responses by leukocyte subsets such as neutrophils and macrophages, to determine the impact of fever-inhibition on leukocyte function, these subsets and their functions would have to be identified. Further, in my study I examined molecular gene expression of a few pro- and anti-inflammatory cytokines involved in inflammation and tissue repair in the site of immune challenge (furuncle). These cytokine profiles could be from effector cell upregulation at the site of immune challenge, or a systemic increase from the hypothalamus to induce effector cell recruitment and functions. Therefore, examination of specific neutrophil gene expression and macrophage gene expression in febrile and fever-inhibited conditions would be a natural next step. Previous experiments showed that a single leukocyte subpopulation such as neutrophils demonstrate phenotypic plasticity and consequently release different soluble factors, cytokines and levels of antimicrobial molecules (ROS/NO) at different points in the acute inflammatory response (Havixbeck et al. 2015). Therefore, I expect that the inhibition of fever attenuates the

earlier responses of leukocytes, resulting in delayed resolution of inflammation, and unsuccessful progression through the phases of tissue repair.

While I was able to quantify the total and subsets of leukocytes at multiple timepoints, another function of immune cells is to increase cell proliferation in order to eliminate a pathogen. In my experiments, I enumerated cell populations at given timepoints in febrile and static conditions however, the use of a BrdU immunohistochemical assay would have allowed for greater resolution on the capacity of fever to alter total immune cell populations through promotion of proliferation. I would expect that the rapid influx of cell infiltration in dynamic conditions is due to an earlier proliferative event compared to static conditions.

5.2.3 Assessment of thrombocyte function within tissue repair

Within this study, I showed the dysregulation of key cytokines in fever-inhibited conditions which was concurrent with delayed tissue repair. Concomitantly, there was an increase in thrombocyte gene expression at later timepoints in fever-inhibited conditions. However, a limitation of this study was that while thrombocyte activation and proliferation were examined, the functional responses of thrombocytes and their role in tissue repair were not examined. This is partly due to the limited thrombocyte function associated genes annotated in the goldfish genome. These limitations can be mitigated by further examination and annotation of thrombocyte genes annotated in other teleost models as many thrombocyte genes show a high degree of homology amongst teleost species. With the examination of thrombocyte functions in goldfish on a molecular level, we may be able to ascertain the effects of fever inhibition on one cell population.

5.3 Relevance

5.3.1 Teleost Health

Fever has been long conserved throughout evolution in endotherms and ectotherms. My work on behavioral fever in a short-term peritonitis model and a long-term tissue repair model contributes another perspective to the ongoing debate. Further study of the febrile response may

not only benefit human health, but also provide an alternative method for reducing inflammation associated die-offs within the aquaculture industry. Expanding our understanding of teleost immunity and the role of fever in teleost health may bring about alternative mechanisms to improve fish health beyond administration of antibiotics.

5.3.2 Strategies in Mitigating Inflammation

Studying febrile responses from an evolutionary perspective and gaining insight into the effects of fever can allow us to harness potential immune enhancing benefits of fever. In human history, it is primarily associated with disease, regarded as a symptom of infection, and subsequently mitigated through the administration of NSAIDs. Within the scientific community, there is ongoing debate on the overall net-positive or net-negative effects of fever. My work highlights the benefits of a natural fever in tissue repair and potential harms in NSAID use in the context of tissue repair. However, currently, there is widespread use of over-the-counter NSAIDs in response to mild or minimal fevers and pain. Expanding our knowledge of the benefits of the febrile response and examining the effects of NSAID use on tissue repair may allow us to gain perspective on pain mitigation strategies that do not delay tissue repair or lead to tissue pathology. Furthermore, the exploration of the impact of temperature on host health in response to tissue injury may lead to alternative strategies in mitigating unwanted symptoms in inflammation.

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