Modulatory Effects of Behavioural Fever on the Inflammatory Reactions of Teleost Fish

by

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Abstract

Since antiquity, fever has been documented as a physiological response to infection characterized by an increase in body temperature. Though fever is commonly regarded as a deleterious symptom of inflammation and is often suppressed using NSAID drugs, little is known about the effects that fever has on immunity due to the difficulty of experimentally blocking fever in traditional endothermic models. Interestingly, ectothermic animals have been seen to undergo a homologous behavioural fever where they exhibit thermoregulatory behaviours to increase body temperature during infection and has been observed in a vast range of distant species including reptiles, amphibians, fish, insects and crustaceans. When combined with traditional metabolic fever the conservation of febrile responses spans over 800 million years of evolution, illustrating the utility of studying fever from a comparative approach using ectothermic models. Many previous studies have displayed strikingly improved pathogen clearance and survival rates in these febrile animals, but few have dissected the mechanisms by which this is achieved.

Previous studies using teleost models have shown that behavioural fever is capable of upregulating pro-inflammatory and antiviral gene expression, but none have assessed the impact of behavioural fever on functional immune responses. To address this gap in knowledge, I used a model of zymosan-induced peritonitis in goldfish to study the effect of behavioural fever on functional inflammatory responses. I found that zymosan injection promoted a behavioural fever consisting of a small increase in temperature preference but drastic decreases in velocity and temperature seeking behaviour, illustrating the use of other 'sickness behaviours' to define febrile responses.

I found that when compared to fish held at normal 16°C housing temperature, the ability to thermoregulate up to 26°C promoted a rapid early influx of leukocytes, and particularly neutrophils, to the inflammatory site. This early cellular response may play an

important role in controlling infections by engaging pathogens early before they can replicate to high levels. In addition, I found that behavioural fever caused the rapid reduction of peritoneal cells to homeostatic levels, but with elevated lymphocyte recruitment two-fold higher than controls. This places fever as an inducer and regulator of inflammation and also a possible regulator of downstream adaptive memory responses. When assessing the antimicrobial responses of inflammatory leukocytes, I found that behavioural fever prevents the high production of reactive oxygen species (ROS), but promotes the induction of inducible nitric oxide responses 24 h earlier than controls. This may play a role in promoting the elimination of pathogens while preventing oxidative tissue damage caused by extracellularly released ROS. In addition, while I found that behavioural fever does not inherently affect leukocytes phagocytic ability, increased *ex vivo* temperature was able to accelerate target internalization in cells derived from both febrile and non-febrile fish, indicating that febrile body temperature likely potentiates phagocytic responses *in vivo*. Interestingly, I found that manually increasing temperature did not replicate immune efficiencies seen in febrile fish, indicating that the regulation of temperature by each individual animal is critical to inducing febrile immunomodulatory effects.

Using a live *Aeromonas veronii* furunculation infection model that mimics naturally occurring teleost infections, I found that thermoregulation promotes the rapid clearance of *Aeromonas* by 7 days (50%) earlier than controls, despite increasing growth rate of *A. veronii* cultures at higher temperatures, indicating that behavioural thermoregulation must modulate host immunity to overcome increased microbial replication rates. Behavioural thermoregulation promoted early iNOS expression, indicating that similar immune-modulatory mechanics were likely taking place within the live infection model. Behavioural thermoregulation also greatly reduced the pathology of induced furuncles and promoted rapid healing of infected lesions. This was associated with early expression of pro-resolution

and healing factors IL-10 and VEG-F, indicating that thermoregulation also actively acts to protect host tissues and potentiate healing responses.

Overall, my data shows that behavioural thermoregulation acts as a potent regulator of teleost inflammatory responses that drives the efficient induction of immune antimicrobial responses that also prevents host tissue damage while promoting tissue repair and an effective return to homeostasis. The ability to control body temperature appears to have evolved as a 'sword and shield' mechanism critical to the efficient clearance of a highly virulent pathogen but also acts to protect against host tissue damage while promoting wound healing to rapidly reach a state of functional tissue homeostasis.

Preface

This thesis is an original work by Michael E. Wong. The research project, of which this thesis is a part of, received research ethics approval from the University of Alberta Ethics Board, as part of a National Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant No. 355303 entitled "Comparative biology of fish phagocytic antimicrobial responses" which was held from 2013-2018. Animal use and care protocols for fish used in this project can be found under Protocol #706 entitled "Comparative biology of fish phagocytic antimicrobial responses".

Dedication

“I have a friend who's an artist and has sometimes taken a view which I don't agree with very well. He'll hold up a flower and say "look how beautiful it is," and I'll agree. Then he says "I as an artist can see how beautiful this is but you as a scientist take this all apart and it becomes a dull thing," and I think that he's kind of nutty. First of all, the beauty that he sees is available to other people and to me too, I believe. Although I may not be quite as refined aesthetically as he is ... I can appreciate the beauty of a flower. At the same time, I see much more about the flower than he sees. I could imagine the cells in there, the complicated actions inside, which also have a beauty. I mean it's not just beauty at this dimension, at one centimeter; there's also beauty at smaller dimensions, the inner structure, also the processes. The fact that the colors in the flower evolved in order to attract insects to pollinate it is interesting; it means that insects can see the color. It adds a question: does this aesthetic sense also exist in the lower forms? Why is it aesthetic? All kinds of interesting questions which the science knowledge only adds to the excitement, the mystery and the awe of a flower. It only adds. I don't understand how it subtracts.”

“See that the imagination of nature is far, far greater than the imagination of man”

-Richard P. Feynman

“Sometimes you have to go on when you don't feel like it, and sometimes you're doing good work when it feels like all you're managing is to shovel shit from a sitting position.”

-Stephen King

The last three-ish years through my Master's degree has been a truly transformative time. I can whole-heartedly say that my ability to critically think and interpret the world around me has grown immensely. If my academic career has taught me anything, it's the perseverance of the human soul in the face of overwhelming failure, and the importance of failure as a tool for learning and growth. Though this journey has often felt long, challenging and to be completely honest insurmountable, it warms my heart to have had the support and encouragement of my peers, friends and family. I couldn't have done it without you all.

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Finally I'd like to thank the National Sciences and Engineering Research Council for funding me throughout my undergraduate and graduate research career, and for providing monetary support for my research project.

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AMPs	anti-microbial peptides
CCL2	CC-chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
cDNA	copy deoxyribonucleic acid
CFU	colony forming unit
DAF-FM-DA	4-Amino-5-Methyl-2′,7′-Difluorofluorescein Diacetate
DAMP	danger associated molecular pattern
DHR	dihydrorhodamine
DNA	deoxyribonucleic acid
dpi	days post-infection
dsRNA	double stranded ribonucleic acid
GFP	green fluorescent protein
GTPase	guanosine triphosphatase
HBSS	Hank's buffered salt solution
HMGB-1	high mobility group 1 protein
hpi	hours post injection
HSP	heat shock protein
ICAM-1	intercellular adhesion molecule 1
IL-6	interleukin 6
IL-10	interleukin 10
IL-1β	interleukin 1 beta
iNOS	inducible nitric oxide synthase
LB	Luria-Bertani broth
LPS	lipopolysaccharide
LXA_4	lipoxin A4
MAMP	microbe associated molecular pattern
MGFL-15	modified goldfish Leibovitz 15 medium
NADPH	nicotinamide adenine dinucleotide phosphate oxidase
NETs	Neutrophil extracellular traps
NLR	nucleotide oligomerization domain (NOD)-like receptor
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	Prostaglandin E-2
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
RIG-I	retinoic acid- inducible gene I
RLR	RIG-I-like receptor
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
T_D	fish allowed dynamic temperature selection within the ATPT
T_{S16}	fish held at static 16°C
TGF-β	transformation growth factor beta

TLR	Toll-like receptor
TMS	tricaine methane sulphonate
TNF	tumor necrosis factor
TSA	trypticase soy agar
VEGF	vascular endothelial growth factor

Chapter 1: Introduction and Literature Review

1.1 Introduction

Fever has long been recognized as a general inflammatory response to myriad infections from viral, bacterial, fungal origins. Currently, fever is often viewed as a symptom of disease rather than a defensive immune response. Thus, fever is often knocked down with anti-inflammatory drugs in clinical settings to prevent discomfort, pathology and mortality in cases of uncontrolled fever. While fever does present danger to neurological systems in extreme cases, there is an expanding body of literature describing the importance of fever as an immune response important to survival against infection, not only in experimental models, but also in clinical settings where the treatment of fever is actually being correlated with increased mortality rates in critically ill patients (1–3). Although the importance of fever has been well documented, many details of how increased body temperature promotes anti-microbial defense and host survivability remain unclear. This is largely due to the difficulty in experimentally manipulating body temperature in classical warm blooded (endothermic) models like mice or rabbits that are often used for immunological research. Febrile body temperature in endotherms can be prevented by lowering ambient temperature, but this technique leads to large amounts of physiological stress generated by an attempt to return to homeostatic set point temperature (4). Some non-steroidal anti-inflammatory drugs (NSAIDS) can be administered to prevent the generation of fever, but also have many off-target anti-inflammatory effects such as preventing human monocyte and lymphocyte activation, inhibiting the production of downstream pro-inflammatory factors and blocking production of functional antibody production in human cells (5–10). These effects may hide or skew any immuno-regulatory effects that may be controlled by body temperature and contribute to the undesirability of using classical endothermic models for febrile research.

Although fever was thought to be restricted to warm blooded animals, a new paradigm of fever was discovered in cold blooded animals, starting with Vaughn's demonstration of bacterially induced behavioural fever in dessert iguanas in 1974 (11). It was shown that infected iguana's would behaviourally increase their body temperature by increasing time spent in warmer areas of an enclosure containing a temperature gradient. Since then, similar febrile phenomena have been found across a vast range of evolution spanning reptiles, amphibians, fish, crustaceans, molluscans, arachnids and insects. Interestingly, an analogous pathogen-induced metabolic temperature increase has also been noted in plants. The evolutionarily conserved nature of these responses hints towards fever as an evolutionary important response to infection. These ectothermic fever responses present an opportunity to comparatively study the impact of fever on inflammatory processes by simply manipulating environmentally available temperatures in comparative models, avoiding the physiological or pharmacological off-target and side effects of manipulating metabolic fever. Comparative models have historically been used to study conserved biological phenomenon, and responsible for several field-breaking discoveries including Nobel Prize winning work by Élie Metchnikoff discovering phagocytosis in starfish larvae (Nobel Prize 1908), or the discovery of Toll pattern recognition receptors in fruit flies by Jules Hoffman (Nobel Prize 2011). These and other comparative studies demonstrate conserved or convergent mechanisms leading to an expanded understanding of mammalian immune systems.

Previously, one of the main hypotheses of how behavioural fever confers increased survivability against many infections was that increasing body temperature simply pushed pathogens past their optimal host temperature leading to decreased proliferation, and therefore improved the capability of the existing immune system to clear the infection (12,13). With advances in molecular techniques and validation of model genomes, several studies have found that behavioural fever induces differential expression of inflammatory

cytokines in an array of fish species including trout and zebrafish. Although mounting evidence supports the theory that fever largely affects host inflammatory responses, little work assessing febrile immunomodulation has been done on a functional cellular level.

Herein, my thesis work employs both an acute peritonitis and live dermal infection in a comparative goldfish model to study the role of body temperature in altering the induction and resolution of inflammation, in addition to the functional inflammatory leukocyte responses expressed by infiltrating leukocytes to elucidate possible underlying mechanisms of febrile immunomodulation.

1.2 Objective of thesis

The objective of my thesis was to dissect underlying mechanisms by which fever potentially alters inflammatory processes to achieve improved host survivability and pathogen clearance by using a comparative behavioural fever model. More specifically, my research focused on the effect of body temperature on leukocyte infiltration kinetics in response to acute zymosan induced peritoneal inflammation, in addition to the functional killing mechanisms exhibited by these infiltrating leukocytes *ex vivo*. A live *Aeromonas veronii* infection was also used to search for differences in pathogen clearance and wound resolution in dermatitis model more representative of inflammatory stimuli encountered in nature complete with host-pathogen interaction and sustained stimuli exposure.

1.2.1 Thesis outline

This thesis is divided into four chapters. Chapter 1 is a review of fever in both endothermic and ectothermic forms, general acute inflammatory physiology and an overview of pathogens employed in my research. Chapter 2 outlines materials and protocols used throughout my experiments. Chapter 3 outlines my work assessing behavioural fever's impact on the induction and resolution of acute inflammatory reactions, specifically to

responding cell responses and functionality. Chapter 4 provides an overview of my findings discusses their relevance along with possible future directions for this work.

1.3 Literature review: Thermoregulation and fever

1.3.1 Introduction

Fever in endotherms is defined as a temporal increase in core body temperature from a set temperature determined by thermoregulatory neurons in the preoptic region of the hypothalamus (14). Although fever is heavily associated as a response to various infections in the medical community, little is known about why this phenomena occurs or what mechanisms it might trigger to alter host defence and survivability. Uncontrolled fever is also associated with increased discomfort, pathology and death, which has led to the commonly prescribed knock down of fever using NSAIDs and possibly preventing the benefits of increased body temperature (15,16). This is largely due to the fact that there are currently no ways of experimentally preventing a fever in traditional mammalian models without the physiological side effects of pharmacological blockade or by inducing hypothermia.

A wide array of ectotherms have been seen to exhibit behavioural fevers, where animals seek out sources of environmental warmth or present with other heat generating behaviours in response to infection or inflammatory stimulus since they cannot increase core body temperature intrinsically (17–22). This presents a unique opportunity to study a widely evolutionarily conserved febrile response by simply allowing or denying animals access to increased environmental temperature. This technique allows the opportunity to study how inflammatory responses might be shaped by body temperature without the caveats of manipulating mammalian fever.

Inflammation occurs when the body detects either a pathogen or host tissue damage and is a highly regulated physiological process that aims to clear invaders or cellular debris

and ultimately return the tissue to its homeostatic functional state. Resident macrophage can detect various molecular patterns from invading microbes and release various chemokines and cytokines to initiate an acute inflammatory response (23). This calls in various inflammatory leukocyte populations including primary responding neutrophils, monocytes and lymphocytes. These cells serve various functions including the direct internalization and killing of microbes through various mechanisms. They also interface with each other to coordinate a controlled shutdown of pro-inflammatory responses and promote pro-resolution responses like tissue repair and remodelling (24).

1.3.2 Fever

1.3.2.1 Endothermic Thermoregulation

In the 1800's, it was identified that a structure in the hypothalamus was responsible for controlling body temperature, though subsequent studies identified a group of structures located in the spinal cord and brain stem that are also responsible for altering body temperature. When these structures are intact, alteration of firing rates in thermoregulatory neurons located in various parts of the hypothalamus including the anterior hypothalamus in addition to parts of the surrounding septum and preoptic nucleus are responsible for maintaining consistent body temperature (14,25–27). Since early studies in the 1900's, these areas have long been known to respond to intracranial temperature fluctuations suggesting that thermoregulatory neurons respond directly to temperature. Conversely, around the 1950s it had been seen in dogs, cats and rats that rapid decreases in environmental temperature decreased extremity temperature, but core body temperature remained unchanged (28–30). This indicated that thermoregulation is also rapidly triggered by sensory input from peripheral sensory to maintain optimal core temperature. More recently, specific warm-sensitive preoptic neurons have been found to respond rapidly both to intracranial temperature and to increased skin temperature, indicating neural integration

of peripheral sensory input with the regulation of core body temperature (31). These warm-sensitive neurons integrate both intrinsic heat generation, as well as behavioural thermoregulatory programs within the nervous system. With the exception of species that undergo states of hibernation or torpor to suppress metabolism to 1% of normal waking rates and body temperature, most endotherms generally maintain a species specific homeostatic body temperature that is vital for consistent biological activity and overall survival (32,33).

1.3.2.1.1 Intrinsic thermoregulatory mechanisms

In response to decreased environmental or body temperature, many adaptive mechanisms exist to raise or maintain core set-point body temperature. Skeletal muscle can be involuntarily exited to cause shivering which can generate heat by increasing mitochondrial respiration rates up to 5 fold in humans (34,35). Further, shivering generates additional heat by replenishment of ATP by oxidation of carbohydrates, lipids and proteins when glycogen stores are depleted (34,36,37). Shivering acts as an acute response to cold stress, but once shivering ceases body heat can also be generated by stimulation of brown adipose tissue (38,39). Present in all mammals, brown adipose tissue is responsible for the vast majority of non-shivering thermogenesis and is likely responsible for the evolution of mammalian endothermy (38). Brown adipocytes are characteristically dense in mitochondria, the majority of which express high levels of UCP-1 (uncoupling protein-1). Also referred to as thermogenin, ablation of UCP-1 in mice prevents non-shivering thermogenesis and prolongs shivering responses for up to 6 months (40). UCP-1 allows mitochondrial combustion of lipids uncoupled to ATP generation which enables a large amount of energy to be chemically released as heat (41,42). Similar uncoupling proteins have also been associated with avian thermogeneration in skeletal muscle cells (43).

Excessively raised body temperature can be extremely detrimental to endotherms by causing protein denaturation and therefore disrupting myriad biological processes. Autonomic nervous responses can trigger a combination of peripheral vasodilation to increase blood flow to the skin and dissipation of body heat from peripheral blood by evaporation via sweat (44,45). Vasodilation plays an important role in maintaining body temperature, as cardiac output to the skin can vary from 2% in hypothermia to up to 60% in states of hyperthermia to either retain thermal energy in the core or radiate it from the extremities to the environment (45). In humans, vasodilation is controlled by various cholinergic nerves originating in the CNS that release the neuropeptide VIP (vasoactive intestinal peptide) and the neurotransmitter acetylcholine, which in combination act to promote vasodilation and perspiration, though exact mechanisms by which this is controlled remain disputed (46–48). Vasoconstriction mechanisms remain less enigmatic, and is largely controlled by endocrine co-transmitters Neuropeptide Y and Norepinephrine released by sympathetic vasoconstrictor nerves (49–51). These factors bind NPY and α-1/2 receptors respectively on cutaneous vascular smooth muscle cells to promote peripheral vasoconstriction (49).

Uncontrolled fever presents a state of elevated body temperature where there is a possibility for severe neurological damage. Due to the perspective of fever as a symptom and not an immune response, there is currently a mentality in both medical communities and lay populations to treat any and all fevers with NSAIDs, despite an increasing body of evidence suggesting that moderate fever is actually beneficial to the host. See sections *1.3.2.3 Suppression of fever* and *1.3.2.3.1 Medical relevance* for more detail.

1.3.2.1.2 Behavioural thermoregulation in endotherms

Although metabolic mechanisms account for the majority of thermoregulation in endotherms, a wide array of behavioural responses to maintain body temperature exist, largely fueled by temperature sensation interpreted in the hypothalamus from peripheral sensory nerve fibers (52–54). These sensations of warm or cold are interpreted and used to achieve thermal comfort, where the environmental temperature allows the return to or maintenance of hypothalamic set-point body temperature (55). The most basic of these behaviours is simply seeking warmer or cooler environments (54). Other warming behaviours commonly found in nature include huddling and sunbathing, while wallowing and seeking shade are used to prevent hyperthermia. Conscience human intelligence has given many more advanced techniques of maintaining body temperature through clothing, shelter and various other technologies that have allowed inhabitation of extreme weather environments despite a lack of biological adaptation for extreme climates (56,57).

1.3.2.2 Endothermic Fever

Fever in endotherms is often considered as controlled hyperthermia characterised by an increase of body temperature set-point which triggers intrinsic thermogeneration by shivering or brown adipose metabolism to raise core body temperature (58). Chills experienced may also contribute to a behavioural thermoregulation, specifically in humans, with heat seeking behaviour such as donning additional clothing or blankets to retain thermal energy regardless of hyperthermic body temperature (12,56). Although raised body temperature is the hallmark of fever, other sickness behaviours can be experienced such as fatigue, lethargy, and lack of appetite.

Classically, fever is generated by detection of foreign microbial products or damaged self by resident tissue macrophages which initiate local inflammation via secretion of cytokines into the bloodstream via diffusion (58,59). Certain cytokines like TNF (tumor

necrosis factor), interleukin 1 and 6 (IL-1 and IL-6 respectively) have been shown to act on meningeal macrophages that line the blood vessels near the ventral preoptic area (60–63). This stimulates expression of cyclooxygenase-2 which catalyzes production of prostaglandin E2 in meningeal macrophages (PG-E2). PG-E2 in turn acts upon thermoregulatory neurons in the preoptic region via localized PG-E2 receptors leading to alteration of temperature set point and downstream thermoregulatory mechanisms (64–66). In murine models, other cells within the nervous system including neurons, astrocytes and microglial cells have been seen to react to inflammatory cytokines by expressing IL-6 which may lead to a positive feedback loop that allows propagation of prolonged febrile responses (67–71).

The classical humoral pathway may not be the only mechanism for triggering fever, as several studies show evidence for the existence of a febrile trigger via the peripheral nervous system (58). In rats, severing of the vagus nerve below the diaphragm, or more specifically to the liver, has been shown to prevent IL-1β expression and PG-E2 production in the pre-optic area and prevents subsequent fever induced by intravenous LPS challenge (72–75). This is significant, as the majority of circulatory LPS is cleared by hepatic macrophages which are thought to be the source of intravenous LPS-induced inflammatory cytokines. In addition, hepatic vagotomy prevents preoptic IL-1β expression, but not its increase in circulating plasma, underlining the importance of peripheral nervous pathways in triggering fever, at least in IV-LPS induced models (58,76).

Once fever has been established, there are several mechanisms that may act to limit or shut down febrile responses. First, temperature itself can reduce the secretion of pro-inflammatory cytokines like IL-1 by up to 90% at 40°C compared to 37°C acting as a feedback loop which may play a role in reducing proinflammatory drivers of fever in meningeal cells (77). Several neuropeptides have also been shown to have antipyretic properties. I will touch on them briefly, but a thorough review by Tatro is available (78). Vasopressin (AVP) is a neuropeptide produced by several groups of central neurons within

the paraventricular nucleus which regulate central autonomic and anterior pituitary pathways throughout structures within the limbic system that also communicate with the hypothalamus (79,80). Perfusion of AVP into the ventral septal area of the brain can prevent the generation of fever in rats, and the neutralization of vasopressin or it's receptor AVP-V1 has been shown to extend the length of fever and increase it's magnitude in rats(81,82). These results suggest that vasopressin plays a role in controlling febrile body temperature and returning the body to a homeostatic temperature. Melanocortins consist of a group of neuropeptides that are generated from a common proopiomelanocortin prehormone which bind a common set of receptors to regulate critical autonomic processes including thermoregulation (83). Primary melanocortin producing neurons are located within the arcuate nucleus and project into various brain stem and fore-brain centers to regulate neuroendocrine and autonomic functions (84). Similarly to vasopressin, administration of various melanocortins has been shown to prevent fever induced by both endogenous cytokines and exogenous inflammatory stimuli in several mammalian species, with particular activity when administered directly to the preoptic or septal regions which maintain high densities of melanocortin receptors (85–90). Blockade of melanocortin receptors by both antibody and artificial inhibitors was also able to increase febrile temperatures (86). Together, these results indicate that temperature and various neuropeptides act to control febrile body temperature and promote the downregulation of febrile hyperthermia to homeostatic body temperatures.

1.3.2.3 Suppression of fever

Since antiquity, the suppression of fever has been a primary therapy for care givers. For example, there is record that before Alexander the Great succumbed to a fever of unknown origin in 323BC, he was treated with cool baths and compresses by Babylonian doctors (91). This technique is still used in modern society, with application of ice baths for

patients presenting with unrelentingly high fever. Similarly, treatment of fever with pharmacological agents has been prevalent in human civilisation, such as the use of willow bark by ancient Egyptians to treat fever documented in the ancient Ebers papyrus dating to 1500 BC (92). In the 1800s, salicin was identified to be the causative agent of these anti-inflammatory, anti-pyretic and analgesic effects (93). By the 1850s, it was chemically altered into the more stable salicylic acid which could be more easily synthesized industrially (94). In 1897 Felix Hoffmann, one of the founders of Bayer's pharmaceutical branch, succeeded in acetylating salicylic acid to reduce gastric irritation caused by the drug (95). Thus acetylsalicylic acid, commonly known as Aspirin, was formed and is still a commonly used therapy after over a century of its discovery (95). Aspirin and other Aspirin-like drugs such as acetaminophen fall into the category of nonsteroidal anti-inflammatory drugs (NSAIDs) and primarily block febrile responses by competitive inhibition of cyclooxygenase (COX) enzymes, of which the isoenzyme COX-2 is responsible for the generation of inflammatory prostaglandins (96–98). This in turn prevents PGE2 production in the pre-optic area of the hypothalamus, inhibiting the generation and maintenance of fever-induced body temperature set-point increase by pro-inflammatory lipid mediators. While the current clinical paradigm prescribes to the suppression of any and all fever with NSAIDs to protect against high-fever associated pathology, there is a growing body of evidence that suggests this practice may be largely detrimental to patient survival. See Section *1.3.2.3.1 Medical relevance* for more detail.

1.3.2.3.1 Medical relevance

Despite growing evidence for the benefits of fever, most medical communities still consider fever detrimental, and the suppression of even mild fevers is commonplace in clinical settings (16). This is likely due to in part the general discomfort caused by fever, but largely to the potential for severe neurological pathology in cases of extremely high fever (99). In clinical settings, anti-inflammatory treatment of sepsis induced fever increased

mortality with patients presenting fevers below 40°C, but improved survival rates in patients presenting higher body temperatures (100). In large correlative clinical studies, pharmacological knock down of fever has also been seen to increase human mortality rates to influenza virus up to 5%, and has also been noted to negatively affect patients in intensive care (2,3,101). Suppression of fever with anti-pyretic drugs has also been seen to increase the load and duration of rhinovirus and influenza virus shedding in both mammalian models and human trials. This trend has also been seen in bacterial infections, increasing the duration of clinical symptoms of induced *Shigella* and *Rickettsia* infections (102–104). On a population level, greater pathogen shedding can lead to an increased opportunity for disease transmission which has long been mathematically correlated to larger outbreak and epidemic sizes (105,106). Combined with the common suppression of fever, these effects may combine to pose a significant threat to public health.

1.3.2.4 Caveats to studying endothermic fever

Although fever has long been recognized as an important physiological response in both evolutionary and medical aspects, our knowledge of febrile mechanisms is constrained by limitations in manipulating endothermic fever generated in classical mammalian models. One method of experimentally dissecting host thermo-immunomodulation is by inducing *in vitro* or *in vivo* whole-body hyperthermia to febrile range using exogenously supplied heat (107–110). This approach, done *in vitro* and *in vivo* allows discrimination of effects gained from increased body temperature alone without the participation of cytokines and lipid mediators generated in febrile responses. The major caveat to this *in vivo* approach is that without true fever response, body temperature is raised above thermoregulatory set point which may induce large amounts of physiological stress that may hide or skew experimental results (111). Thermoregulatory stress induced through the hypothalamic-pituitary-adrenal axis can have many distal effects in immunity including immunosuppression by glucocorticoids like cortisol, which can alter leukocyte trafficking and downregulate pro-

inflammatory cytokine expression (112,113). Glucocorticoids are also capable of mediating T cell helper type 2 (Th2) responses in lymphocytes by inhibiting pro-Th1 cytokines like IL-12, IL-2 and TNFα while promoting pro-Th2 IL-10, IL-4, IL-13 production to switch from cell mediated to humoral adaptive responses, protecting tissues and organs from further cell mediated damage or stress (114). Alternatively, adrenergic stimulation can promote not only activation of pro-inflammatory NFκB pathways in non-activated leukocytes, but also pro-resolution effects on previously activated leukocytes (115). These examples demonstrate part of the myriad pressures that stress can have on shaping immune reactions, and thus may be impacting research employing hyperthermia. *In vitro* cell line studies can be used to observe thermal responses to individual cell types, but lack nuanced interplays between leukocyte sub-populations, other tissue cells and distal soluble factors that may be critical in modulating those cell types in the context of an active inflammatory site or infection.

Another way of manipulating endothermic fever is through pharmacological suppression using anti-pyretic drugs. These present a way of inducing inflammatory responses while inhibiting febrile induction but inherently perturb inflammatory programs through off target effects. *See Section 1.3.2.3 Suppression of fever*.

1.3.2.5 Behavioural thermoregulation and fever

While fever has been noted throughout thousands of years of history in endotherms and humans in particular, it has only recently been noted within the last 40 years that cold blooded animals share a similar thermoregulatory response triggered by infection and inflammatory queues. Since ectotherms generally lack mechanisms for intrinsic thermogeneration, they induce fever by displaying various behaviours to alter body temperature. In 1974, the Vaughn and others were the first to demonstrate behavioural fever experimentally by inducing increased temperature preference in desert iguanas (*Dipsosaurus dorsalis*) that were injected with gram negative *Aeromonas hydrophila*, but

not sterile saline (11). Since this seminal discovery, equivalent thermoregulatory behaviours have been noted in various other ectothermic species including other reptilians, amphibians, fish, crustaceans, molluscans and insects (18–22,116–142)(*See Figure 1.1 for a phylogenetic tree, and Table 1.1 for a list of species and respective immunological stimuli*). Many of these organisms thermoregulate by simply moving to warmer temperatures, but other behaviours like sunbathing or flattening to increase conductance also contribute to these behaviours. More complex social behaviours have been noted specifically in bees, which will move to warmer parts of the hive in low grade infections, but can also increase whole hive temperature using friction to induce a colony wide fever in hive outbreak conditions (19,143,144). When grouped with traditional endothermic fever, these febrile responses consistently appear across 800 million years of evolution. Interestingly, analogous febrile responses have also been documented in plants, where string bean (*Phaseolus vulgaris*) leaf temperatures increased to 6°C from 2°C over ambient temperature when infected with a fungal pathogen, likely by increased respiration rates (4,145) (Figure 1.1, Table 1.1). When included as an analogous response to endothermic and behavioural fevers, febrile responses have evolved across an astonishing 1.5 billion years of evolution divergence, indicating that they likely play a pivotal role in the survival of myriad organisms (4).

While behavioural fever in fish has only been demonstrated in laboratory settings, behavioural fever has been shown to be protective in wild populations of frogs and lizards infected with fungal or parasitic pathogens respectively (20,120). Further, many behavioural studies found that behavioural fever increased temperature preferences by only a few degrees, making it highly likely that behavioural fever is employed in natural settings, especially in habitats with deeper waters that allow great temperature variation through the presence of a thermocline (131–134,146).

These responses can be separated into two categories: behavioural thermoregulation and behavioural fever, which may not be mutually exclusive phenomenon. Behavioural thermoregulation may induce organisms to seek out a warmer homeostatic temperature in the absence of immunological challenge, while behavioural fever, characterised by a temporal increase in temperature preference driven by infection, may overlap with homeostatic thermoregulation and partially obscure any clear 'window' of febrile thermopreference. Though thermoregulation plays a part in behavioural fever as a mechanism by which ectotherms must increase their body temperature to 'febrile' ranges, increases in body temperature alone generally do not yield the same immuno-kinetics as seen in various models of behavioural fever, including my own research outlined further in this thesis (See Results and Discussion sections) (131,142). Many of these models report prostaglandin triggers and other intracranial inflammatory responses that are also present in endothermic fever, suggesting that behavioural fever lies between generic thermoregulation displayed by ectotherms and true metabolic fever responses induced in endotherms, including humans (127,132,134,136,147–149). This evolutionarily conserved response of increasing body temperature specifically during infection allows for the comparative study of true febrile immunomodulation using models of behavioural fever in cold blooded animals.

There are both beneficial and detrimental effects of moving to warmer areas, particularly in aquatic organisms. Firstly, achieving an increased body temperature is metabolically expensive, with a 11-13% increase in oxygen metabolism over every 1°C increase which will cause the animal to expend much more energy (150,151). This upregulation in metabolism is paired with oxidative stress, but is counteracted by a suite of stress-induced factors like heat shock proteins that act to protect host tissues. Increased oxygen consumption through increased metabolism may be particularly taxing on aquatic life, as oxygen solubility in water decreases with increased temperature which may limit

oxygen availability to the organism (152). Thermoregulating to warmer regions of water such as shallows or near surface waters may also increase the rate of predation and thus decrease the survivability of animals in these areas. However, increasing temperature may also cause certain microorganisms to die or lose virulence which may positively impact the course of infection (94). Regardless of any potential detrimental effects, there must be a net benefit for the widespread conservation of behavioural fever, which likely lies in it's ability to vastly improve pathogen clearance and survival rates across various species of hosts and pathogens.

1.3.2.6 Thermoregulation in fishes

Body temperature of fish species are largely governed by their surrounding waters, due largely to water's high conductance and specific heat capacity, or the amount of energy that is required to increase water temperature (153,154). These factors lead to large exchanges of thermal energy through circulating blood under the skin and especially at the large un-insulated gill interface which make it incredibly challenging to retain body heat above ambient temperature (154). In addition, almost all documented fish lack brown adipose tissue responsible for the majority of mammalian heat generation, and thus over 99.9% of known fish species remain purely ectothermic and poikilothermic (having large core temperature ranges) (154–156). Consequentially, studies of over 250 species of aquatic organisms from different taxa have revealed a widespread active selection of optimal thermal zones for each species within their environments (153). These temperature zones are often correlated with optimal growth, feeding, and behaviours of their respective species (157,158). However, depending on the species, thermal preference may alter due to season, age, time of day, acclimation temperature, and other physiological states as well as social and environmental interactions. In addition, not all members of a species may adhere strictly to a collective thermal preference (153,159).

Although most fish are purely ectothermic, there are categories of fish that have evolved adaptive mechanisms to generate and/or retain body heat to maintain a higher body temperature than ambient water, at least temporarily. These include lamnid and thresher sharks, manta rays, swordfish, billfish, mackerels and tuna (154). These animals are continuous swimmers that generate heat by slow-oxidative muscle fibre thermogeneration and reduce heat loss through multiple mechanisms (154). Firstly, thermogenic muscle fibers are located deeper within the muscle and are thus insulated from external temperatures by overlaying tissues (155,160). In addition, vessels arranged into countercurrent heat exchange configurations allow conservation of heat from outgoing warm blood by energetic transfer to incoming cool blood from the extremities (161). Billfishes and some mackerel have also evolved specialized heat generation tissues within the cranium, which along with endothermic brown adipose tissue, are the only known tissues to exist for the primary purpose of endothermy (162,163). In contrast to uncoupled mitochondrial respiration in mammals, heat is generated in these tissues by continuously cycling calcium ions between cytoplasm and sarcoplasm through a Ca^{++}ATPase in muscle cells that lack contractile myofibrillar machinery (164–166). Though these animals can generate or retain heat for periods of time, many maintain only locally increased temperatures likely to provide buffered visceral, nerve or optic temperature to retain functionality at depth (167–169). These animals lose heat retention over time and are eventually required to thermoregulate behaviourally. This was demonstrated in bigeye tuna and swordfish that rapidly gain high body temperature by surfacing before increasing muscular thermogeneration and shunting blood away from heat exchangers to absorb environmental energy (170). Internal body temperatures then slowly decline to 18°C as these fish dive 500-1000 meters to feed below the thermocline in cold, nutrient rich water (170–172). Since these animals generally maintain elevated temperatures in only specific areas of the body, they are identified as regional endotherms.

Recently, an instance of whole body endothermy was demonstrated in the large deep-water moonfish *Lampris guttatus*, also referred to as the opah or Jerusalem haddock (173). While these fish lack specialized heat generation organs, they acquired many adaptations to allow heat generation and retention to permit perfusion of the whole body with warm blood to maintain a constantly elevated body temperature without the need for behavioural thermoregulation. The main adaptational difference between opahs and regional endotherms are its tightly stacked counter-current exchanges within fatty-insulated gill arches that warm cold oxygenated blood with warm de-oxygenated blood. This greatly reduces the amount of energy lost at the gill-environment interface, and when coupled to thermogeneration by unusually large pectoral muscles used as the main propulsive muscle group, allows *L. guttatus* to maintain elevated temperature body temperature while remaining below the ocean thermocline. Specialization to this cold environment highlights evolutionary adaptation to allow permanent niche expansion into nutrient rich waters while retaining high ocular, neural, aerobic and other physiological functions that allow sustained high performance predation (173). While it is possible that these fish might be able to induce an intrinsic febrile response, this ability in opahs have yet to be assessed.

1.3.2.7 Immune modulation by behavioural fever

Since Kluger *et al*. reported that behavioural fever in desert iguanas increased survivability of *Aeromonas hydrophila* infection by 75%, several subsequent studies have also noted correlations between increased thermopreference and survivability in various species including crickets, locusts, toads, zebrafish, carp and goldfish (128,131,132,140,142,174). While this protective correlation exists across a vast evolutionary span, the mechanisms that drive pathogen clearance and survival rates remain relatively unknown. Boltaña *et al* of the Mackenzie group addressed this functional gap in knowledge by assessing transcriptome changes in zebrafish stimulated with dsRNA which mimics a viral infection (132). They found that febrile animals under simulated viral

infection induced a wide array of genes several fold higher than static temperature controls. These genes include members of the tripartite motif containing (TRIM) family, involved in anti-viral RIG-I signalling and viral capsid binding, interferon regulatory factory IRF7, and a suite of interferon inducible factors, helicases and resistance genes all of which are heavily involved in anti-viral responses (132,175–177). Further, Rey *et al*, also of the Mackenzie group further supported the anti-viral effects of behavioural thermoregulation in zebrafish using larval organisms. They again displayed temperature dependant upregulation of anti-viral genes including Grass Carp Reovirus-Induced Gene 2 (GIG-2), TRIM25, IRF7, Myxovirus resistance protein B (MxB) and Viperin which is a potent promoter of anti-viral genes (133,178). More recently, behavioural fever has been seen to protect carp from mortality induced by a strain of cyprinid herpesvirus mutant in a soluble TNF-α decoy receptor. When this mutation was restored, behavioural fever was blocked which significantly increased viral replication, pathology and mortality of fish (142). This displays the targeting of febrile responses as an important virulence factor and lends further support to the role of fever as an important response in pathogen defence. Although these results hint heavily to immune modulation by fever, no evidence exists addressing how behavioural fever might alter the functional activity of responding leukocytes in ectotherms.

1.3.2.8 Temperature effects on the regulation of microbial gene expression

Though increasing amounts of data indicate that fever heavily influences immune function, body temperature can also have direct effects on invading microbes to alter the balance of host-pathogen interactions. Variation in temperature leads to alteration of not only microbial physiological and metabolic processes, but may also greatly alters the paradigm of microbial gene expression (179–181). For example, many pathogens of endotherms have host-temperature dependent expression of virulence factors such as adhesion molecules, secretion systems, toxins, iron chelators and immune evasion factors that are required during infection but are otherwise energetically expensive and not useful

for environmental exposure or within a vector host (181,182). This makes temperature a critical factor for microbial survival within a host that may alter its body temperature in response to infection (179,181).

Temperature dependent gene regulation in bacteria can be mediated by a variety of mechanisms at all regulatory steps of expression. For example, changes in DNA supercoiling can increase or decrease DNA accessibility, and bending of promoter sites can alter RNA polymerase binding to Shine-Dalgarno sites to prevent transcription (183–187). Additionally, histone-like proteins can bind mRNA in a temperature dependent manner to silence them by preventing ribosome association and thus protein expression (188,189). Ribosomal binding sites may also be hidden within mRNA temperature dependant secondary structure, or bound by trans-acting small blocking sRNAs that melt at specific temperatures to allow ribosome binding and thus translation of the transcript (182,190–193). These small RNAs in particular are extremely sensitive to temperature and allow gene regulation from small temperature fluctuations. Another way that bacteria have been shown to regulate temperature sensitive genes is by thermo-labile repressor proteins. These factors have a wide range of structural features, but all have specific on/off temperatures where protein conformations are changed to allow either binding or release of DNA sequences to alter gene expression (179). A huge range of temperature dependent genes have been identified, though these are mostly within mammalian and especially human pathogens (180,182,194,195).

1.3.2.8.1 Gene regulation in aquatic pathogens

Due to medical relevance and the potential for pharmacological targeting, the emphasis of study surrounding temperature dependent regulation in microbes has been primarily around mammalian and specifically human facultative pathogens that generally have clear planktonic-pathogenic gene expression paradigms that turn on at host body temperature (179,180). Consequentially, temperature regulatory

systems and their outcomes in aquatic pathogens remains largely unclear, especially given the complex nature of body temperature and its similarity to the surrounding environment in most exothermic aquatic hosts. Although some of the mechanisms described above in Section *1.2.3.8 (Temperature effects on the regulation of microbial gene expression)* may be important for heat shock responses, it is unlikely that they play large roles in engaging virulence factors in aquatic pathogens, again due to their similarity to environmental temperature. Regardless, there are similar temperature dependent mechanisms that are more likely to play a role in host-environment expression paradigms.

One possible mechanism of temperature dependent expression could also involve short non-coding RNA sequences (181). These differ in action and act by binding non-coding 5' untranslated regions of specific mRNAs to stabilize them and effectively prolong their abundance and translational availability (196). This type of regulation is used in a global bacterial stress response powered by RNA polymerase sigma factor S encoded by the *rpoS* gene. *RpoS* transcripts can be bound by Downstream region A (*DsrA*) sRNAs to stabilize and increase translation (196,197). Not only is *DsrA* expression dependent on temperature, but also the stability of *DsrA* transcripts themselves, allowing for fine-tuned physiological responses to fluctuating temperatures (196,197). Some protein regulators of early infection have been seen in human pathogens that mechanistically may play a role in temperature regulation of exothermic pathogens. Since tissue invasion often starts in extremities or upper respiratory tracts where body temperatures hover around 30°C, many pathogens have a set of regulators that are active around this temperature to promote virulence factors important for early infection such as flagellar motility in *Legionella monocytogenes* and tissue colonization factor invasin in *Yersinia pestis* (198,199). The invasin coding gene is bound by Regulator of Virulence protein A (*RovA)* at 25°C

which enables its transcription. In later stages of infection at 37°C, the RovA protein changes conformation and is targeted for protease degradation, shutting down invasin expression (200). A similar process occurs with *L. monocytogenes* GmaR-MogR complex, which dimerize at 30°C to promote *flaA* flagellar expression, but dissociate at 37°C to not only prevent *flaA* expression, but also actively represses it by binding of non-complexed MogR (201). This type of regulation may be important mechanism to toggle various virulence factors and environmental adaptations, especially if they have a broad host specificity that includes both endo and ectotherms. With this in mind, since most ectothermic body temperatures are similar to those in the immediate environment, it is likely that temperature alone is not responsible for a large portion of virulence expression like it is in endothermic pathogens, but more likely a combination of temperature with other physiological states found within the host, but not the environment.

1.3.3 Detection of inflammatory stimuli

1.3.3.1 Pattern Recognition Receptors

In order for an innate inflammatory response to be generated, mechanisms must be in place for the detection of situations that require the induction of inflammation. The first innate receptor found to respond to bacterial components and trigger production of anti-microbial factors was the *Drosophila melanogaster* Toll receptor, discovered by Jules Hoffmann's group in 1996 (202). Homologous Toll-like receptors (TLRs) were identified in mice, in which Bruce Beutler's lab demonstrated that TLR-4 mediates inflammatory responses to lipopolysaccharide (LPS) stimulation (203). These joint discoveries opened the door to a huge field of ongoing study surrounding immune activation mechanisms and garnered a shared Nobel Prize in Medicine and Physiology in 2011. Since this seminal discovery, a large number of TLRs in addition to analogous and homologous receptors have

been identified in a vast range of species across evolution from plants to mammals (204,205). These receptors can be found in membrane bound or cytosolic forms and commonly respond to both conserved microbial components or host-damage associated motifs to trigger respective inflammatory programs through transcription factors like NFκB, interferon regulatory factor and activating transcription factor. Pattern recognition receptor (PRR) groups in mammalians include TLRs, C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene I like receptors (RLRs) (206). Although many of these receptors and their ligands have been identified in mammals, fish do not share all the same receptors, or their functionality. For example, zebrafish have 20 TLR variant genes, only 10 of which are orthologs of the 13 TLR genes found in humans. In addition, not all fish homologs retain mammalian receptor activity, such as TLR-4, which does not respond to LPS stimulation as it does in mice (207). Receptor variants generally occur by gene duplication, though in teleost or bony fish, a third full genome duplication, and an evolutionarily recent fourth duplication in cyprinid fish including carp and goldfish, provide the opportunity for further TLR expansion (208,209). Several fish specific TLRs have evolved largely within the TLR11 family with TRL 20 and TLR 22 and TLR 23 being identified in a wide variety of fishes and some other non-mammalian vertebrates like *Xenopus* (210). Many of these genes have been duplicated in piscine species with several TLR size variants noted in goldfish, rainbow trout and Atlantic salmon, the agonists for these receptors is still relatively unknown (211,212). TLR 22 has been identified to recognize double stranded RNA in fugu pufferfish, suggesting that this lineage of TLR might play an analogous role to human TLR3 which senses double stranded viral RNA on cell surfaces (213). Although PRRs may bind only singular motifs, many use adapter molecules such as TLR4 and MDA binding LPS, or may oligomerize with other receptors to alter ligand specificity (214).

1.3.3.2 Inflammatory molecular patterns

1.3.3.2.1 Microbe-associated molecular patterns

Formerly known as pathogen associated molecular patterns (PAMPs), microbial associated molecular patterns (MAMPs) are evolutionarily conserved motifs that are vital for function and survival of various pathogen groups including bacteria, fungi, viruses and protozoan parasites. These factors should not be present within host tissues and their presence is indicative of infection. Some MAMPs include structural cell wall components such as LPS, peptidoglycan motifs, microbial lipoproteins, envelope proteins and β-glucans (215–220). Various nucleic acids present a second group of targets generated primarily through viral genome replication and includes double stranded RNA, ssRNA within the lysosome, and unmethylated CpG DNA (221–223). Other conserved motifs that act as TLR ligands include virulence factors like bacterial flagellin which is important for motility of flagellated pathogens (224). The physiological importance of these factors and their evolutionary conservation allows a small number of PRRs to detect and thus launch an inflammatory response against a wide array of microbial invaders.

1.3.3.2.2 Damage-associated molecular patterns

Although the theory that immune systems detect 'non-self' motifs to trigger inflammation dominated early ideology, building evidence lead Polly Matzinger to suggest an alternative 'Danger theory' where the host responds to self-ligands exposed by tissue damage (225). The recent body of research suggests that both theories are true, and that Danger or Damage-associated molecular patterns (DAMPs) that are passively released or actively secreted by damaged host cells can stimulate immune reactions (226,227). A large amount of these molecules are motifs that are normally sequestered from extracellular exposure and are classified as "hidden self". Upon necrotic death or lysis, released intracellular factors such as

genomic DNA, various heat shock proteins, uric acid, mitochondrial DNA and peptides, and ATP (228–231). Some released factors can function indirectly by altering pre-existing extracellular molecules like collagen, hyaluronic acid, heparan sulphate or fibrillary protein into pro-inflammatory mediators (232–235). Other factors within the cell may also activate clotting factors or complement which generate further pro-inflammatory mediators like anaphylatoxins C3a, C4a and C5a (227). Interestingly, in rats and humans, complement driven inflammation may also be triggered by detection of released cytoskeletal, non-muscle myosin heavy chains by a self-reactive IgM antibodies forming immune complexes that trigger classical complement cascades. Together, these self-damage detection strategies allow a wide array of ligands and mechanisms for redundant detection of uncontrolled cell death to mediate efficient initiation of inflammatory programs.

1.3.3.3 Zymosan detection by dectin-1 and TLR2

The main pathogen mimic used to generate infection in my research is zymosan, which is an integral component of yeast cell walls. Zymosan consists of cell wall protein-carbohydrate complexes, the majority being β-glucans comprised of glucose moieties connected by β-1,3-glycosidic linkages, and is regularly isolated from baker's yeast *Saccharomyces cerevisiae* (236,237). In mammals, zymosan binds to the C-type lectin receptor dectin-1, which upon complexing with β-glucans can signal through recruited SYK (spleen tyrosine kinase) kinases to mediate phagocytosis and respiratory burst responses in macrophages (238–240). However, dectin-1 signalling in mammals does not appear to produce significant changes in cytokine expression, indicating that dectin-1 promotes mostly functional leukocyte responses, and not propagation of inflammatory triggers (241). To this end, immune surveillance against β-glucans is two-fold, with TLR-2 also directly binding zymosan and signalling through MyD88 pathways to promote downstream inflammatory cytokine expression through NFκB (238,240,241). Carp macrophages have been seen to

bind zymosan directly to generate similar reactions to mammalian counterparts including ROS, NO and cytokine generation, particularly IL-1β and IL-11 (239). Our lab has demonstrated similar results in goldfish primary macrophages, which can internalize zymosan coated particles and generate high levels of ROS (242–244). Additionally, zymosan injection can induce cytokine generation and high levels of leukocyte recruitment *in vivo*, similar to responses seen in mice (243). Thus, it is likely that fish also detect zymosan through conserved dectin-1/ TLR-2 pathways, or analogous receptors that produce similar functional outcomes.

1.3.4 Leukocyte kinetics of acute inflammation

Leukocyte recruitment is a key step involved in generating a local inflammatory response. Various cell subsets are attracted to the site by cytokines and chemokines generated by resident macrophages upon detection of microbial or damage associated signals. While many specialized leukocyte subsets found in mammalian systems have not been identified or do not exist in teleost fish, goldfish do have neutrophils, monocytes, macrophages, and lymphocytes which constitute main cellular lineages that contribute to mammalian acute inflammation (243). These cells not only act to seek out and destroy microbes, but also to clear apoptotic and necrotic cells from the inflammatory site in an effort to return the tissue to a functional homeostatic state.

1.3.4.1 Leukocyte recruitment by resident phagocytes

Tissue resident cells such as macrophages or dendritic cells act as sentinels, alerting the immune system to local insults by recruiting and activating additional inflammatory leukocytes. Inflammation is triggered upon detection of damage or microbial associated molecular patterns (DAMPs or MAMPs respectively) through respective TLRs or other innate PRRs. These can be derived from various microbial cell components, or intracellular components of host cells released upon necrotic cell death (222,238,245–247). Signalling induces the expression of downstream cytokines and chemokines which will cause

upregulation of adhesion molecules by endothelial cells to allow cells within the circulation to slow and roll along the vessel wall until they can transmigrate into the site (248). Cytokine production also promotes chemo-attraction and activation of respective cell subsets towards the inflammatory site. Although these pathways are innate, and thought of as relatively non-specific, cross talk of various signals can tailor the inflammatory response to respond to different infection types, such as intracellular vs extracellular pathogens (246,249). More details on PAMPs, MAMPs, PRRs and their signalling capacity can be found in *Section 1.3.3*

1.3.4.2 Recruitment of neutrophils

Neutrophils are known as potent mediators of anti-microbial activity and are among the first responders in an inflammatory site. They are attracted early in inflammatory responses by CXCL8, platelet activating factor, and the lipid mediator leukotriene B4 (248,250). In humans, neutrophils are recruited from the peripheral blood where they make up to 50-70% of circulating leukocytes (251,252). While goldfish contain only 5% peripheral leukocytes at homeostasis, they are recruited instead from the head kidney (the hematopoetic organ of teleost fish), and increase to 50% of blood leukocytes shortly after intraperitoneal injection with zymosan (244). These neutrophils will bind E and P selectins expressed by activated endothelial cells through P-selectin glycoprotein ligand and E-selectin ligand-1 respectively to mediate close contact rolling in the high flow-through environment of the blood stream (248). This allows close association of neutrophilic surface integrins with intercellular adhesion molecule 1 (ICAM-1) which stops cell rolling and along with various other receptors promotes the crossing of the endothelium from the bloodstream to an active inflammatory site, also known as diapedesis (248,253). In goldfish, neutrophil infiltration to the site peaks at around 18 hours post injection (hpi) and reduces over 24-48hpi to homeostatic levels at 72hpi (244).

Once neutrophils have entered the site and become activated, they can deploy a range of cellular weapons to kill microbes that have entered host tissues. This includes phagocytosis, production of highly reactive oxygen and nitrogen species, and the release of toxic granule components. Neutrophils are also capable of a cellular kamikaze termed NETosis, that invert the cell into an extracellular trap (254). The cells' exposed histones and chromatin trap bacteria and help kill bacteria along with various antimicrobial peptides (AMPs) and proteases which are present in high quantities in mammalian NETs (255,256).

Although neutrophils are traditionally thought of as only pro-inflammatory mediators, there is building evidence that they also play a role in controlling the resolution of inflammation. Recently, it has been identified that like humans, late inflammatory goldfish neutrophils are capable of producing lipoxin A4 (LXA_4) which reduces ROS production in macrophages, and has been seen to increase phagocytosis of apoptotic bodies (243,244,257). Internalization of apoptotic neutrophils by macrophages in turn promote the expression of pro-resolution cytokines, like IL-10, which promotes further downregulation of inflammation and the switch to inflammatory resolution by various mechanisms (258).

Traditionally, it was thought that all neutrophils undergo apoptosis once they're no longer needed, but more recent work in zebrafish has shown that while some neutrophils become apoptotic, others can reverse-transmigrate back into the blood stream (259). While the activity of these cells after migration is unknown, it appears that egress of neutrophils from an inflammatory site may at least play a role in reducing local inflammation to a homeostatic level.

1.3.4.3 Recruitment of monocytes

In mammals, classical monocytes are an extremely plastic subset of leukocytes which originate from the bone marrow and mobilize into the blood stream to move into tissues and differentiate into a range of dendritic cells or macrophages during homeostasis, although newer evidence suggests that monocytes only develop into mature tissue

macrophages during developmental primary hematopoiesis, which then seed host tissues and self-replicate to maintain pools of resident tissue cells (260–262). In addition to their role in cell replenishment, classical monocytes also to respond to acute inflammatory sites where they can mediate antimicrobial defences including phagocytosis and subsequent generation of ROI and RNI species or phagolysosome fusion (263).

In humans and mice, monocytes can be guided into the blood stream and various tissues through surface C-C chemokine receptor type 2 (CCR2) sensing gradients of C-C-chemokine ligand 2 (CCL2, also known as monocyte chemotactic protein-3). Even though CCR2 is critical for monocyte trafficking to infection sites, other chemo-attractants than CCL2 also play roles in attracting monocytes to sites of infection (264,265). Neutrophils play a large role in recruiting monocytes using factors released during degranulation like azurocidin which promotes monocyte-endothelial adhesion and acts as a chemoattractant for monocytes along with granular LL-37, cathepsin G, and human neutrophil peptides 1-3 (266,267). In addition, various granule-derived proteases degrade extracellular matrix components which allows easier movement of monocytes into the tissue. Once monocytes have been attracted to the endothelial expressed vascular cell adhesion molecule 1 (VCAM1), various factors like P and E-selectins, CCR1 and CCR5 play a role in binding various chemokines and are critically involved in slowing monocyte movement in the blood stream and promoting subsequent transmigration into tissues (265,268).

1.3.4.4 Resolution and a return to homeostasis

Once the microbial challenge has been cleared, cell levels in the inflammatory site, specifically the large influx of neutrophils, will decline to basal levels. In both mammalian and fish models, this paradigm shift involves a switch in lipid mediator production from the pro-inflammatory leukotriene B4 (LTB4) to the pro-resolution lipoxin A4 (LXA4) (244,269,270). It was long thought that neutrophils only play pro-inflammatory roles and undergo apoptosis after microbial elimination before being cleared by macrophages. This

hypothesis is being adjusted, as *in vivo* imagine of zebrafish neutrophils show only a small portion of neutrophils undergoing apoptosis in the inflammatory site, with the majority of them 'reverse migrating' back into circulation (271). Other studies have found that these cells can persist for several days, and can travel between wounded tissue and circulation multiple times, however the purpose for this extended trafficking is unknown (272). Neutrophils that do terminate within the inflammatory site undergo the classical stages of apoptosis while secreting factors including various, lysophosphatidylcholine, sphingosine 1-phosphate and fractalkine that are chemoattractant to scavenger cells (273). These factors allow macrophage and dendritic cells to find apoptotic cells while surface expression of apoptotic signals like phosphatidylserine, annexin 1 and calreticulin promote internalization and clearance of apoptotic bodies (274). Uptake of apoptotic bodies by macrophages promotes inflammatory resolution two-fold: by downregulating expression of pro-inflammatory cytokines while upregulating expression of pro-resolution cytokines like interleukin 10 (IL-10) and transformation growth factor β (TGF-β) (275). Late neutrophil derived LXA4 further promotes uptake of apoptotic neutrophils (276). Resolvin E1, another pro-resolving lipid mediator acts to attenuate monocyte, macrophage and dendritic cell NFκB activity through chemerin receptor 23 binding (277).

Once inflammatory cells have been cleared, tissue remodelling and a return to homeostatic functionality must take place. This process is immensely complex and requires tight regulation and cross-talk between many subsets of cell types. Recent work has highlighted alternatively activated macrophages as a key mediator of wound healing. Macrophages produce growth factors like IL-10, TGF-β and vascular endothelial growth factor (VEGF) which promote proliferation of neighboring tissue cells (278). These factors act to regulate extracellular matrix remodelling, promote collagen synthesis, formation and repair of blood vessels, and fibroblast differentiation among other processes that allow the functional reconstruction of wounded or inflamed tissues.

1.3.4.5 Lymphocytes

Lymphocytes play an important role in vertebrate adaptive immunity, mediating antigen specific responses that allow for 'memory' of past infections and improved defence upon repeat challenges. Although divisible into ever expanding subcategories, two main lineages of lymphocyte exist (279). T cells, characterized by the expression of recombinant T cell receptors that traditionally are responsible for cell-mediated immunity, and B cells, which are characterized by their recombinant B cell receptor which can be secreted in soluble antibody form that mediate humoral based adaptive defences. These cell specific receptors are constructed from immunoglobulin (Ig) domain conserved heavy chains that determine isotype with light chains which undergo somatic recombination of variable, diversity and joining gene domains (280,281). This recombination can generate an enormously diverse array of receptor structures from relatively few genes, with 10^{11}-10^{15} combinations possible depending on species and receptor type (282,283). Higher vertebrates like mammals generate robust memory responses by aggregation of lymphocytes with antigen presenting cells in lymph nodes and germinal centers where they can undergo secondary recombination and immunoglobulin class switching to select better antigen specificity and functionality by altering antibody isotype from IgM or IgD to IgG, IgA or IgE in mammals (284,285). Isotypes are generally associated with various anatomical sites that they protect such as serum (IgG), mucous (IgA) or skin (IgE). Due to the evolution of the immunoglobulin system in a common ancestor of cartilaginous fish and vertebrates, many features of fish adaptive responses are similar, although only three isotypes have been identified in teleosts. These include IgM, IgD and IgTeleost/Zebrafish (IgT/Z), the latter of which appears to be the dominant isotype involved in mucosal immunity which is critical in aquatic environments (286,287). Regardless of this fact, fish adaptive immunity and thus vaccine responses are typically poor compared to higher vertebrates which may be due to several factors including a lack of major germinal centers that promote lymphocyte maturation such as lymph nodes. Teleosts have less developed

lymphoid associated tissues in the gut and gills, though none have been identified near skin which makes up a large area of un-keratinized mucosal tissue (288–290). Additionally, class determination of antibodies is terminal in fish, with no potential for class switching available. These patterns are clearly seen through evolution with the development of adaptive features becoming more prevalent from cartilaginous fishes through amphibians, birds and higher mammalian vertebrates (291).

1.3.5 Soluble inflammatory mediators

Cytokines represent a family of intercellular messengers that work in a complex, fluid network to control and balance immune reactions. They are generally small proteins, secreted by a variety of cells including leukocytes, fibroblasts, endothelial and other tissue cells that can function via autocrine, paracrine and endocrine routes. While cytokine effects on individual cells or sites culminates from the summation of many cytokine effects, certain factors play larger roles in the induction and control of inflammation. While some functions may not have been shown in fish, all major classes of cytokines are present in teleost fishes, with highly conserved structures and many functional homologs, paralogs or convergent analogs being described throughout vertebrate evolution (292). Herein, I will briefly review the function of some soluble factors that play major roles in acute inflammation.

1.3.5.1 Pro-inflammatory mediators

1.3.5.1.1 IL1-β

Interleukin 1-β is a member of the beta trefoil cytokine or Interleukin-1 family which are central to the regulation of inflammation. IL1-β was the first interleukin described in teleost fish, though multiple copies exist that can be classified into two groups based on genetic organisation: Type 1 which consists of seven intron/exon regions conserved among jawed vertebrates and Type 2, with a five or 6 intron/exon regions that appear to have arisen from an ancestral IL1-β gene precursor (293–

295). In my research, I focus on IL1-β1, as it holds functional similarities to mammalian IL-1β, allowing closer comparative analysis.

IL1-β is released by a variety of cells types after inflammatory activation through receptor sensing of PAMPs or DAMPs (296). Like many other cytokines, IL1-β is synthesized as a pro-protein that requires either intra or extracellular cleavage to become biologically active, allowing safe storage but rapid deployment of pro-inflammatory mediators (297,298). Transcription factors of downstream PRR signalling like NFκB also promote d*e novo* pro-protein synthesis to rapidly replace cytokine stores, allowing the approximate measurement of cytokine deployment by quantification of gene expression (299). Biologically active IL1-β is capable of potent upregulation of other inflammatory cytokines like TNF-α, and IL-17 in fish, mediated by binding to IL-1 receptor complexes and downstream signalling through the highly conserved NFκB pathway (292,300,301). IL-1β can recruit inflammatory cells by both acting directly as a leukocyte chemoattractant and also by promoting the production of other chemoattractant cytokines (chemokines) such as CXCL-8, a specific recruiter of neutrophils (302,303). While IL1-β is foremost described as a pro-inflammatory mediator, it has also been shown to induce expression of pro-resolution cytokines such as IL-10 and TGF-β (304,305). This early expression of pro-resolution cytokines likely plays a role in both preventing uncontrolled inflammation and acts to mediate the kinetics of inflammatory resolution within the site.

1.3.5.1.2 TNFα

Named after the characteristic triple layered β-sheet 'jelly roll' topography of it's protein monomers, the Jellyroll or tumour necrosis factor cytokine family is an extremely ancient group of inflammatory mediators homologs traced to invertebrates (306–309). My research focuses on tumour necrosis factor α (TNFα) which is divided into three groups in fish: Type 1 with conserved synteny with human TNFα and is

studied in my research, type 2 which are teleost specific, and TNF-N, which has yet to be characterised (310). Within these groups, multiple paralogs have been reported across a variety of teleost species including goldfish (311,312). While various fish TNFα isoforms appear to facilitate similar pro-inflammatory mechanisms as human TNFα *ex vivo*, there are variable expression rates of different isoforms across TNFα types in various cell groups, tissues and circumstances which indicates that they might play slightly different roles depending on inflammatory context (309).

TNFα is produced as a transmembrane pro-protein and is bioactive in both its membrane bound form and after conversion to soluble forms via cleavage of both transmembrane and N-terminal intracellular domains by TNF-α converting enzyme (310,313,314). Soluble TNFα appears to be bioactive in monomeric, or homo-dimeric or trimeric forms in fish. While receptor binding is less clear in fish, mammalian TNFα signals through TNFα receptor 1 and 2 to facilitate a wide range of pro-inflammatory effects (306,308,315–317). Many of these effects overlap IL-1β functionality, as they share common signalling through inflammatory transcription factor NFκB (310). This includes induced expression of additional TNFα along with other pro-inflammatory cytokines like IL-1β, IL-8, IL-17, and genes involved in inflammatory lipid mediator synthesis (310,315,318). Like IL-1β, TNFα promotes leukocyte infiltration by acting both directly as a chemoattractant and via induced expression of other chemotactic factors (316,318). TNFα also promotes various antimicrobial effects and has also been seen to directly increase leukocyte phagocytic capacity and ROS production in fish cells (312,319). In both mammals and fish, TNFα has been seen to facilitate pro-apoptotic activity in some cell types, but also upregulates expression of granzymes which are used by cytotoxic cells to induce apoptosis in target cells (306,320,321).

These mechanisms may synergise to promote improved removal of infected cells within the inflammatory site.

1.3.5.2 Pro-resolution mediators

1.3.5.2.1 TGF-β

Transformation growth factor beta is a member of the cysteine knot cytokine family named after a beta sheet structure rich in disulphide bonds, of which other members include a wide range of growth and differentiation factors (322–324). This cytokine is an ancient and conserved immune-regulatory factor, with homologs present throughout vertebrates and invertebrates (325). While three homologs exist throughout vertebrate species, TGF-β1 is the dominant form expressed within the immune system, all three variants induce similar effects *in vitro* (326). Like many other cytokines, TGF-β is produced as a pro-protein, and is released extracellularly in its bioactive soluble form after cleavage of its N-terminal domain (327). These forms can bind a suite of TGF-β superfamily Type 1 and 2 receptors to mediate immunosuppressant and wound healing effects (323).

In fish, TGF-β1 functions to control inflammation through suppression of induced pro-inflammatory cytokine expression like IL-1β TNFα, IL-8 and IL-6. Along with suppression of the iNOS gene, recombinant TGF-β has also been seen to functionally downregulate production of nitric oxides (328,329). TGF-β has also been seen to control IL-1β protein availability by induction of a soluble IL-1β receptor that acts as a molecular decoy, further downregulating pro-inflammatory action (330). Along with immunosuppressive activity, TGF-β alone can also induce proliferation in peripheral blood leukocytes and fibroblasts, but blocks proliferation induced by LPS (328,330,331). These opposing mechanisms likely act to replenish cells late in inflammatory reactions while preventing expansion of additional inflammatory leukocytes.

In mammals, TGF-β also plays extensive rolls in myriad regulatory processes including the differentiation of T cells, particularly to T_h2 or T_{reg} lineages, recruitment of monocytes, generation of collagen by fibroblasts, reduction of mast cell activation and suppression of natural killer cell cytolytic capacity (323). While no corresponding evidence exists in fish, due to the conserved nature of TGF-β, it is likely that some of these tissue-protective functionalities are also conserved in teleost species.

1.3.5.2.2 IL-10

Interleukin 10 is an immunosuppressive member of the functionally diverse type 2 alpha helix cytokine family and is present across teleosts in a single gene copy (309,332). IL-10 is known to mediate several immunosuppressive or pro-resolution effects and is crucial in controlling inflammation and preventing inflammatory pathology and autoimmune diseases (333). Cell type specificity of IL-10 expression in fish remains relatively unknown, but nearly all subsets of mammalian leukocytes are capable of IL-10 expression under various contexts (333). Binding of soluble IL-10 to heterodimeric IL-10R1 and 2 complexes signal through the Janus kinase and two signal transducer and activator transcription protein (JAK-STAT) pathway to mediate its effects (334). In fish, IL-10 has been seen to mediate a robust set of pro-resolution effects like downregulating expression of pro-inflammatory cytokines such as IL-1β, TNFα CXCL-8 and components of the NADPH oxidase which generated reactive oxygen species (335). Along with cytokines, IL-10 can also downregulate the expression of MHCII in carp, while also increasing IgM concentrations in a vaccination setting (336). IL-10 has myriad functions described in specific cell subsets in mammals, but its importance as an immunosuppressive factor is displayed by the use of IL-10 homologs by viruses to supress host immunity, creating favourable environments for prolonged infection and replication in both humans (Epstein-Barr virus) and fish (Koi herpesvirus) (337,338).

1.3.5.2.3 VEG-F

Vascular endothelial growth factors (VEG-F) are members of the platelet-derived growth factor supergene family that play various roles including embryogenesis, the development of vascular and lymphatic systems, inflammatory or pathophysiological angiogenesis and the maintenance of newly formed blood vessels (339–342). These glycoproteins are secreted as dimers that are stabilized by 8 conserved cysteine residues that form di-sulphide bonds which act to stabilize both secondary and tertiary protein structures. These dimers bind with varying specificities to a series of VEGFR receptor complexes that signal through various tyrosine-kinase domains (343). I focus on VEGF-A in my thesis as it plays a crucial role in inflammatory neovascularization and tissue repair (344). In mammals, the majority of VEGF-A is generated by alternatively activated or wound-phenotype macrophages, which are mostly derived from infiltrating monocytes, although other cell types like keratinocytes, endothelial cells, fibroblasts, platelets and neutrophils have also been shown to express VEFG-A to lower levels (345–349). VEGF-A promotes endothelial cell migration and proliferation to facilitate new vessel formation (344,347). It also promotes chemo-attraction of additional macrophages, and is involved in the formation of lymphatic vessels (350). VEGF-A has been identified in teleost fish including zebrafish and fugu pufferfish where it has also been seen to play an important role in angiogenesis (351,352). This is unsurprising, as there is a high level of sequence homology between fish and mammalian VEGF-A with 68-70% amino acid homology (353).

1.3.5.3 Chemokines

While many cytokines can act as chemo-attractants to various cell types, chemokines are a subdivision of cytokines that act primarily as major chemo-attractant mediators that

direct cells to various sites during development and homeostasis but also recruit cells to the inflammatory site during various stages of inflammation.

1.3.5.3.1 CXCL-8

Interleukin 8 (IL-8), also known as CXCL-8, is a potent inflammatory mediator belonging to the CXC chemokine family which are named after their two N-terminal cysteines separated by a single nucleotide (354). Members of this family, and particularly CXCL-8 are potent recruiters of neutrophils for which it is also coined as neutrophil chemotactic factor and neutrophil activating protein (355,356). CXCL-8 exists in bioactive monomers or dimers, both of which bind primarily through CXCR-1 and activate various intracellular cascades through G protein adapters (357,358). While the major roll of CXCL-8 is to recruit and activate neutrophils, it is also chemotactic for monocytes, lymphocytes, and other granulocyte subsets within the inflammatory site (355). The dimeric form of CXCL-8 can also bind to glycosaminoglycans on endothelial cells to promote angiogenesis, but also to present dimeric CXCL-8 to neutrophils circulating in the bloodstream to draw them into an inflammatory site (358,359).

Along with recruiting neutrophils to the site, CXCL-8 also activates neutrophils through a wide variety of mechanisms. Activation with CXCL-8 can induce upregulation of integrins that promote neutrophil interaction with the endothelium and subsequent rolling and diapedesis into inflammatory sites (360). CXCL-8 also activates various anti-microbial mechanisms including the generation of respiratory burst and release of neutrophilic granules that contain various proteolytic and antimicrobial proteins (354,361). Because of its role as a potent recruiter and activator of neutrophils, a number of chronic inflammatory diseases that are associated with overexpression of IL-8 including rheumatoid arthritis, psoriasis, and various pulmonary and cardiac inflammatory diseases (354).

A single homologous CXCL-8 genes has been identified in all teleost fish species that have been assessed, while some species like zebrafish and carp contain two distinct isoforms which both retain homologous functionality which are capable of chemo-attracting neutrophils at 200ng/mL concentrations *in vitro* (362). Zebrafish CXCL-8 has been particularly well characterized, as they've become important models for tracking the movement of leukocytes, and largely neutrophils, *in vivo* due to the transparency of larval fish (363–365).

1.3.6 Microbial killing mechanisms

1.3.6.1 Phagocytosis

Phagocytosis is the process by which cells 'eat' or internalize particles from their surroundings and is a deeply conserved mechanism critical not only to the clearance of foreign microbes, but also in the development and maintenance of homeostatic tissues. The seminal discovery of phagocytosis and phagocytic cells in starfish larvae by Élie Metchnikoff in the 1800s birthed the field of modern cellular immunology while displaying the utility of comparative biology (366). Phagocytosis is employed generally by 'professional phagocytes' which include monocytes, neutrophils, osteoclasts and a wide array of macrophage and dendritic cell types (262). I will focus on immune functions of phagocytosis as it pertains to inflammatory sites and infections.

Phagocytosis can be engaged by myriad receptor types that allow detection of ligands which signal to phagocytes that a particle is either foreign or damaged. The goal of internalization is two-fold: as a mechanism to kill invading microbes or to clear apoptotic or necrotic host cells in an effort to return the tissue to a homeostatic state (367). When pathogens are internalized inside a phagocytic vacuole, host lysosomes (granules) containing anti-microbial hydrolytic enzymes and oxidases fuse to the vacuole to form the phagosome (368). In macrophages, this environment is highly acidic, whereas neutrophilic phagosomes are neutral which likely allows for robust NADPH oxidase activity and high ROS

production seen in neutrophils compared to macrophages (369). Depending on cell type, many factors can be released from granules into the phagosome including cytolytic antimicrobial peptides, reactive oxygen and nitrogen species (257). Neutrophils present the majority of phagocytes in an acute inflammatory site and can engulf and kill microbes very quickly. This speed makes the first wave of cellular responders very efficient at clearing microbes, but also causes tissue damage and additional inflammation due to premature phagosomal maturation and the premature release of granular contents into incomplete phagocytic vacuoles and subsequent release to extracellular space (370). Despite being an important tool for clearing invading microbes, phagocytosis is also involved in pro-resolution reactions. Once neutrophils have cleared an infection, a portion of them will become apoptotic and release lipoxin A4, which calls monocytes and macrophages to internalize neutrophilic apoptotic bodies which downregulates expression of CXCL-8 and other inflammatory responses in both mammals and fish (244,276).

1.3.6.2 Reactive oxygen species

While reactive oxygen species (ROS) are also used in intracellular signalling networks, I will focus on its use as an antimicrobial mechanism (371). The generation of ROS is associated with cellular interaction and phagocytosis of immunologically stimulating particles, and is often termed 'oxidative burst' due to its associated high increase in oxygen consumption (372). ROS production is controlled by the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) complex. Activation requires inducible translocation of $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ subunits to the cytosolic face of the membrane where they form the functional oxidase with membrane resident $gp22^{phox,}$ $gp91^{phox}$ subunits and Rac GTPases (257,372). During phagocytosis, cytosolic membrane containing the oxidase complex will form the inner facing wall of the phagosome, allowing the NADPH oxidase to form reactive O_2^- radicals that can damage microbes directly, or though subsequent reactive products including hydrogen peroxide and hydroxyl radicals (371,373). These products act through

various chemical reactions to attack lipids, proteins and DNA that can neutralize internalized microbes, but can also damage host tissue if released extracellularly (374)

1.3.6.3 Nitric oxide generation

Immunological nitric oxide (NO) is generated by conversion of L-arginine and oxygen to L-citrulline and NO by inducible nitric oxide synthase (iNOS), or NOS2 (375). NOS2 is a flavoprotein containing a heme group that forms a functional homo dimer, and is distinct from the other two types of nitric oxide synthases (neuronal and endothelial NOS), in that its expression is entirely turned off in non-activated cells and becomes induced by pro-inflammatory stimuli like PAMPs or cytokines (376,377). This inducible nature and bio-activity is well characterized in mammals and is also well conserved in fish (378). Inducible NO production has mainly been assessed in neutrophils and macrophages, though the latter is known to functionally produce much higher levels of NO. In these cell types, bio-active iNOS has been seen to aggregate in various granules, near phagosomes, in small vesicles and attached to cytoskeletal components below the cytoplasmic membrane (379–384). Labile NO radicals that are produced can have many destructive effects on proteins and other cellular components either directly, or through formation of downstream reactive molecules like nitrosothiols, superoxide anions and peroxynitrite. These compounds can damage proteins by various mechanisms including thiolsilation of cysteines which disrupt disulphide bridges and nitration of tyrosines residues (385,386). Enzymatic function of many proteins can also be inhibited by high affinity binding of NO to catalytic copper and zinc cluster cores, many of which are involved in electron transport and metabolism (387). NO also has damaging effects on nucleic acids, with low levels causing deamination of nucleotides and inducing mutations, and high levels leading to strand breaks and fragmentation of DNA (388,389). Together, these effects contribute to the functional inhibition and destruction of microorganisms by NO.

iNOS genes in fish share the same inducibility patterns to similar triggers that are found to induce mammalian iNOS including certain cytokines like IL-1, IL-4, TNF and interferons suggesting conserved use of NO as an antimicrobial response across vertebrate species (390). Further, NO production in both human and goldfish monocyte cultures is delayed for several days until differentiation into mature monocytes or differentiation into macrophages (391).

1.3.6.4 Neutrophil degranulation

Like other granulocytes, neutrophilic granules are packed with a variety of antimicrobial factors including oxidases that generate reactive oxygen and nitrogen species, small antimicrobial peptides and proteases that can be deployed against invading microbes. Although there are 4 main types of granules in mammalian neutrophils, I will focus on the general components and effects of granular contents and degranulation as the field concerning composition and physiological effects of specific granules is an increasingly complex and expanding area of interest. This subject has been recently reviewed in detail by Cowland and Borregaard (361). The primary target for granules is to fuse with phagosomes and deliver granular contents to engulfed microbes, facilitating antimicrobial activity. However, rapid neutrophilic engulfment of microbes and subsequent fusion of granules to incomplete phagosomes may allow the release of granular components into the extracellular milieu causing further inflammation and host tissue damage (257,392). Direct degranulation can also occur during 'frustrated phagocytosis', where a neutrophil cannot engulf a target, generally due to size, and will secrete granular contents directly onto the pathogens surface, creating a large amount of collateral host tissue damage and inflammation in the process (257).

Although granules are generally thought to be involved in antimicrobial responses, release of specific granules upon neutrophil diapedesis also allows the presentation of adhesion molecules on the plasma membrane and degradation of the extracellular collagen

matrix, improving recruitment speed of responding neutrophils to the inflammatory site (250).

1.3.6.5 AMPs

Antimicrobial peptides (AMPs) are a diverse class of small molecules that have been identified in all forms of cellular life that have been assessed, from prokaryotes to humans (393). Though this group of proteins includes well defined bacterial antibiotics, I will focus on vertebrate AMPs which exhibit broad non-specific antimicrobial activity and are important to innate immune defense. These AMPs are small proteins, commonly less than 12 amino acids in length that contain one positively charged end with a large proportion of hydrophobic residues on the opposing side when folded into bioactive forms (394). AMP function is conserved by structure and can be separated into four groups: α-helical, β-sheet, loop and extended AMPs (395–398). These proteins commonly act by being drawn to and incorporating into negatively charged bacterial membranes to disrupt membrane structure and function by forming transmembrane pores, collapsing membranes into micelles with protein aggregates, or to cross the bacterial membrane to interact directly with intracellular bacterial enzymes (399,400). While many amps are constitutively expressed in many tissues and fluids, many are held in pro-protein form within granules, where cleavage can be induced to release bioactive AMPs (393).

In addition to their antimicrobial properties, more recent evidence suggest a heavily immunomodulatory role of AMPs. One well defined example is the human cathelicidin LL-37, which has been shown to recruit neutrophils while reducing their apoptotic activity, altering expression of various pro-inflammatory cytokines and promoting monocyte differentiation into inflammatory macrophages (401,402). Similar effects have been seen with several human neutrophil peptides (HNP) which are present in high concentrations at neutrophil degranulation sites (403). This demonstrates the importance of AMPs not only to direct

microbial clearance, but also to tailoring of the inflammatory reactions in which they're deployed.

1.3.7 Aeromonas

Aeromonas is a genus of Gram negative, rod shaped bacteria which can cause a wide range of infections relevant to both human health and aquaculture. Aeromonads can be found ubiquitously throughout the environment in water and soil, and can be carried by fish, aquatic invertebrates, amphibians, insects, birds, livestock and pets (404).

1.3.7.1 Relevance as aquatic pathogens

Aeromonas species have long been associated with disease in fish, particularly in aqua-cultural settings. *A. salmonicida*, *A. hydrophila* and *A. veronii* are largely responsible for Aeromonad diseases in farmed fish and can cause a wide variety of local or systemic pathologies. Local dermal infections can cause ulcerative disease like furunculosis, though acute infections involving septicemia can cause hemorrhaging of muscle tissues and internal organs (405,406). Disease due to *Aeromonas* outbreaks can cause mass die offs in economically important species like salmon, trout, tilapia and carp which amount to hundreds of millions of dollars in loses per year (407).

1.3.7.2 Relevance in Public Health

Although *Aeromonas sp.* have long been associated with disease in fish, they are being increasingly acknowledged as an opportunistic pathogen associated with human disease. Because of their ubiquitous presence, they are a common contributor to gastroenteritis in both developing and developed nations contracted through the consumption of contaminated water or food (408,409). Aeromonad gastroenteritis is especially prevalent in diarrheal disease in children and travellers (410,411). Like most opportunistic pathogens, Aeromonads are also known to cause septicaemia and further disseminated disease such as vital organ failure in immunocompromised patients (404).

Septicemia of immunocompetent patients can also occur, but is usually associated with penetrative trauma that inoculate the bacteria or allow entry into the body such as cuts, bites or crush injuries (412). Near drowning events can also cause pneumonia related sepsis, and burns from fire or explosions can become inoculated by local water sources which are often used to extinguish flames (413,414). These types of wound-induced infections can also develop into necrotising fasciitis which can lead to amputation (415). *Aeromonas sp* are also known to cause urinary tract infections in clinical settings, and are also associated with corneal ulceration and keratitis due to unsanitary handling and maintenance of contact lenses (416–419).

1.3.7.3 Aeromonas veronii virulence factors

Aeromonas veronii is the most pathogenic of the major *Aeromonas* species, with *A. veronii* biovar sobria being the most pathogenic strain. This biovar has been isolated from goldfish held in the University of Alberta aquatics facility, and is used in my research to induce live dermal infection. Analysis of other *A. veronii* isolate genomes indicate that they carry a large array of virulence factors that allow them to mediate invasion and infection of a diverse range of hosts, though not all factors are present across the species (420–422). Virulence factors in *A. veronii* can be segregated into five basic groups: adhesins or structural components, extracellular factors including toxins and lytic enzymes, secretion systems to deliver these products, iron sequestering systems and quorum sensing.

1.3.7.3.1 Structural components

Structural components like flagella play critical roles in the movement of bacteria through liquids and are used in related pathogenic *A. piscicola* strains to invade host cells, though this activity has not been assessed in *A. veronii* (423). In addition, flagella can aid in biofilm anchoring, and is complemented by fimbriae like the bundle forming pilus (*Bfp*) which also facilitates adhesion to human cells (424). Outer membrane proteins (OMPs) also facilitate binding to host cells, but also serve

to form a membrane A-layer which protects bacterial membranes from attack by soluble host defences in addition to their functions in nutrient uptake, osmoregulation and signalling (425,426). A poly protein-sugar matrix capsule that covers the bacterial membrane also serves a similar function in conveying resistance to humoral immunity while promoting cell invasion (427,428).

1.3.7.3.2 Toxins

Aeromonas veronii secretes a variety of enzymes and toxins that facilitate various pathogenic effects important for establishment and virulence within the host. A major portion of these enzymes include a wide range of proteases, lipases and DNases that act to degrade mucous layers and host tissues to promote invasion and establishment of infection (421,426). B-Hemolytic toxins such as hemolysin A (*hlyA*) and the species specific aerolysin (*aerA*) are strongly associated with the development of lesions and mortality in fish (429). *A. veronii* also uses cytotoxic (*act*) and cytotonic (*alt,ast*) enterotoxins to kill host cells or alter their morphology to permit further pathology (430). Expression of these toxins appear to be temperature dependant, likely due to the obligate lifestyle and wide host specificity of *A. veronii* (422). Other less characterized toxins with unknown function are associated with virulence, such as a protein with sequence homology to the repeats-in-toxins (RTX) family of bacterial toxins.

1.3.7.3.3 Secretion systems

A series of secretion systems is used for many functions, including the delivery of toxins either extracellularly or intracellularly. Type II secretion systems are responsible for extracellular secretion of various enzymes and toxins outlined above, in addition to acting as a general secretion pathway for other nutrient chelators and quorum sensing factors (431–433). Type III injectisome and IV secretion systems are both used to deliver enterotoxins into the cytosol while the

Type IV system is also responsible for bacterial conjugation and the exchange of genetic material. This becomes incredibly important in the transfer of virulence factors and especially antibiotic resistance genes between bacterial strains in aquacultural and hospital settings (434–436).

1.3.7.3.4 Iron sequestration

Like most pathogens, iron acquisition systems play an important role in for survival in the host where most iron is sequestered. *Aeromonas* species secrete a vast array of siderophores that bind iron with high affinity, and respective receptors than can scavenge iron-bound moieties (426). However, siderophores are incapable of removing iron from haemoglobin, which is an abundant source of iron in the host. An additional set of haeme-binding proteins can accomplish this to access additional iron along-side siderophore systems (437,438).

1.3.7.3.5 Quorum sensing

Quorum sensing factors can be released, sensed and responded to by bacteria to act as a communication system which allows for the coordination of biofilm formation, expression of virulence factors, antibiotic production, and plasmid conjugation (434,439–441). In *Aeromonas sp* a *Vibrio*-like autoinducer synthetase (*luxl*) is responsible for generating acylated homoserine lactones that act as quorum agents through AHL receptors (441–444).

1.3.7.3.6 Temperature effects on Aeromonas virulence factors

Though many temperature-dependant expression systems have been identified in bacteria, many function to induce virulence factors to mediate endothermic invasion and infection by inducing gene expression at 30-40 degrees (181). These systems seem unlikely to function in pathogens of ectothermic hosts which retain a body temperature similar to that found in the environment. Though for this reason it is likely that pathogens of ectotherms regulate virulence expression

through other sensory systems, some temperature related effects have been noted in *Aeromonas* species. Yu *et al* assessed *Aeromonas hydrophila* expression at various temperatures via Maldi-Toff analysis and found that the expression of several virulence factors like flagellins, S-layer, serin-metaloprotease, hemolysin and the Type III secretion system were much higher at 25°C compared to 37°C (445). Consequentially, the secretion of virulence factors occurs to a much higher degree at lower temperature, despite the optimal growth temperature of *A. hydrophila* being around human body temperature. This pattern of upregulated virulence factor expression at environmental temperature has been observed in several other important aquatic pathogens including *Yersinia ruckerii* (causative agent of red mouth disease in salmonids), *Flavobacterium psychrophilum* (causative agent of bacterial cold water disease in salmonids), *Lactococcus garvieeae* (causative agent of lactococcosis in fish), *Vibrio harveyi* (bacterial white tail disease in shrimp), *Vibrio salmonicida* (cold water vibriosis) and *Edwardsiella tarda* (Edwardsiellosis or hemorrhagic septicemia). (446–455)

1.4 Summary

While fever has long been acknowledged as a symptom of infection since antiquity, relatively little is known about how this induced increase in body temperature might modulate innate cellular immune reactions. Utilizing the well conserved nature of fever across nature, we will gain knowledge of possible immunomodulatory mechanisms that are triggered by behavioural fever in bony fish. Using this comparative approach, we hope to uncover mechanisms that may also be modulating inflammatory responses in mammalian fever to further our understanding of immunity.

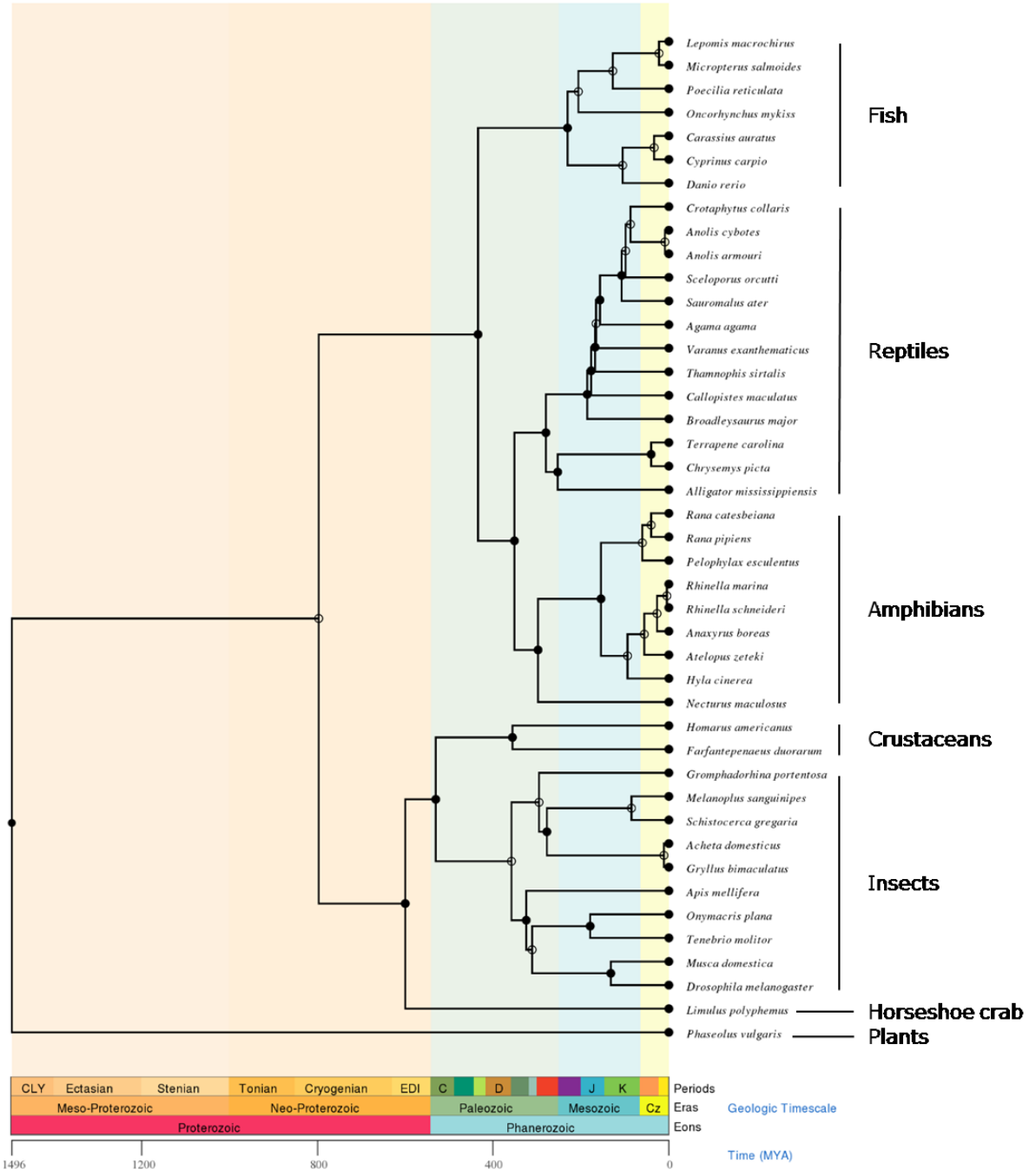


Figure 1.1 Phylogeny of organisms described to exhibit behavioural febrile responses. Genus and species of organisms shown to exhibit increased behavioural thermopreference upon various immune stimulation listed in the current literature. Evolutionary time scale listed in millions of years ago with black dots indicating current species or common divergence points between species and clades. Phylogenetic tree generated using timetree.org distance calculator and its associated databases. Eight species in the literature were unresolved due to updated phylogeny of respective species and were thus excluded from the figure. See Table 1.1 for a full species list and references. Geologic Periods, Eras and Eons in addition to time (millions of years ago) displayed below. Coloured bars correspond to time periods.

Table 1.1 Organisms that exhibit behavioural fever and their corresponding immunological stimuli. A list of all ectothermic organisms (at time of writing) that have been reported to display behavioural fever and the immunological stimulus that induced respective febrile responses**.**

Common Name	Species	Stimulus	Ref.
Bluegill sunfish	*Lepomis macrochirus rafinesque*	*Aeromonas hydrophila*	(130)
Large-mouth blackbass	*Micropterus salmoides*	*Aeromonas hydrophila*	(130)
Trinidadian guppy	*Poecilia reticulata*	*Gyrodactylus turnbulli* (helminth ectoparasite)	(135)
Rainbow trout	*Oncorhynchus mykiss*	Lipopolysaccharide	(134)
Common goldfish	*Carassius auratus*	*Aeromonas hydrophila,* lipopolysaccharide	(131)
Common carp	*Cyprinus carpio*	Cyprinid herpesvirus 3	(456)
Zebra fish	*Danio rerio*	Spring Carp viremia virus, dsRNA	(132, 133)
Nile Tilapia	*Oreochromis niloticus*	*Streptococcus iniae*	(457)
Collared lizard	*Crotaphytus collaris*	killed *A. hydrophila*	(116)
Hispaniolan trunk-ground anoles	*Anolis cybotes*	*Eutrombicula alfreddugesi* (chigger mites)	(120)
Hispaniolan trunk-ground anoles	*Anolis armouri*	*Eutrombicula alfreddugesi* (chigger mites)	(120)
Granite spiny lizard	*Sceloporus orcutti*	killed *A. sobria (veronii)*	(123)
Common chuckwalla	*Sauromalus obesus (Sauromalus ater)*	*Aeromonas sobria (veronii)*	(119)
Red-headed rock agama	*Agama agama*	killed *A. sobria (veronii)*	(123)
Savannah monitor	*Varanus exanthematicus*	killed *A. hydrophila*	(117)
Common garter snake	*Thamnophis sirtalis*	*Aeromonas sobria (veronii)*	(21)
Dwarf tegu	*Callopistes maculatus*	killed *A. hydrophila*	(117)
Sudan plated lizard	*Gerrhosaurus major (Broadleysaurus major)*	killed *A. hydrophila*	(117)
Green anole lizard	*Anolis carolinensis*	Lipopolysaccharide	(122)
Common box turtle	*Terrapene carolina*	*Aeromonas hydrophila*	(118)
Painted turtle	*Chrysemys picta*	*Aeromonas hydrophila*	(118)
American alligator	*Alligator mississippiensis*	Lipopolysaccharide	(121)
American bullfrog tadpoles	*Rana catesbeiana*	*Aeromonas hydrophila*	(124)

Common Name	Species	Stimulus	Ref.
Northern leopard frog	*Rana pipiens*	*Aeromonas hydrophila*	(124)
Edible frog	*Rana esculenta*	Prostaglandin E1, *mycobacterium sp.*	(126)
Cane toad	*Rhinella (Bufo) marinus*	Lipopolysaccharide	(125)
Schneider's toad	*Rhinella schneideri (bufo paracnemis)*	Lipopolysaccharide	(129)
Western Toad	*Anaxyrus boreas boreas*	*Batrachochytrium dendrobatidis* (amphibian chytrid fungus)	(128)
Panamanian golden frog	*Atelopus zeteki*	*Batrachochytrium dendrobatidis*	(20)
Green tree frog	*Hyla cinerea*	killed *A. hydrophila*	(458)
Salamander (common mudpuppy)	*Necturus maculosus*	Prostaglandin E1	(127)
American Lobster	*Homarus americanus*	Prostaglandin E1	
Pink shrimp	*Penaeus duorarum*	Prostaglandin E1	(136)
Great Ram's Horn snail	*Planorbarius corneus*	Lipopolysaccharide, zymosan	(459)
Madagascar cockroach	*Gromphadorhina portentosa*	*Escherichia coli*	(137)
American crayfish	*Cambarus bartoni*	*Aeromonas hydrophila*	(460)
Grasshopper	*Melanopus sanguinipes*	*Aeromonas hydrophila*	(139)
Dessert locust	*Schistocerca gregaria*	Metarhizium anisopliae var. acridum (fungus)	(140)
Senegalese grasshopper	*Oedaleus senegalensis*	killed *Metarhizium flavoviride*	(461)
House cricket	*Acheta domesticus*	*Rickettsiella grylli* (prokaryotic intracellular parasite)	(174)
Field cricket	*Gryllus bimaculatus*	*Rickettsiella grylli* (prokaryotic intracellular parasite)	(462)
Common yellow scorpion	*Buthus occitanus*	Prostaglandin E1	(138)
Southern man-killer scorpion	*Androctonus australis*	Prostaglandin E1	(138)
Honey bee (individual and colony)	*Apis mellifera*	*Nosema ceranae* (microsporidian) Ascosphaera apis (fungi)	(19,143)

Common Name	Species	Stimulus	Ref.
Namib desert tenebrionid beetle	*Onymacris plana*	Lipopolysaccharide	(141)
Yellow mealworm	*Tenebrio molitor*	Lipopolysaccharide	(463)
House fly	*Musca domestica L.*	*Beauveria bassiana* (fungal pathogen)	(464)
Fruit fly	*Drosophila melanogaster*	*Metarhizium robertsii* (fungal pathogen)	(465)
Ribbon leech	*Erpobdella obscura (Nephelopsis obscura)*	Lipopolysaccharide	(18)
Atlantic horseshoe crab	*Limulus polyphemus*	Prostaglandin E1	(136)
Common string (French) bean	*Phaseolus vulgaris*	*Colletotrichum lindemuthianum* (fungus)	(145)

Chapter 2: Materials and Methods

2.1 Animals

2.1.1 Fish

Carassius auratus auratus (common goldfish) were purchased through Aquatic Imports in Calgary, Alberta at approximately 15cm in length. These fish were held in opaque 16°C continuous flow-through aquaria where they were acclimatized for at least 14 days after transport. Fish were continually monitored for disease and were fed twice daily. Prior to experimental handling, animals were netted and anesthetized in a 50mg/L tricaine methane sulphonate (TMS) solution. Fin clips were performed for identification purposes only when necessary. Animals were sacrificed via cervical dislocation as approved by Animal Care and Use Committees and Science Animal Support Services. All efforts were made to ensure animals experienced the minimal, necessary stress required for experimentation, and termination procedures were carried out efficiently.

2.2 Annular temperature preference tank

The annular temperature preference tank (ATPT) was originally designed by Myrick *et al* to determine temperature preferences in aquatic organisms (466). The ATPT is constructed from acrylic and consists of three concentric rings: an outer-most inflow ring separated into 8 equal segments around the periphery, a continuous swim chamber containing no physical barriers, and an inner circle to control depth, outflow and drainage. Small equidistantly drilled pores placed high on the inflow chambers and low on the outflow chamber allows for the radial flow of water from the periphery to the center of the apparatus. This generates a ring shaped swim chamber that has constant depth, current and perceived cover that may alter fish behaviour. Mixing of various water temperatures within the inflow chambers allows the generation of 8 temperature zones maintained by fluid dynamics. Maintenance of these zones was verified using a dye test to track fluid

movement from input chambers, across the swim chamber and into the drainage area of the apparatus with tight specificity to respective zones and little mixing into neighboring zones (Figure 2.1). The temperature of 12°C and 16°C water lines are maintained by the University of Alberta Aquatics facility while 16°C water is fed through a Chronomite SR-40 electrical flow through heater to reach 40°C water used to generate and maintain the desired zone temperatures. Temperatures of various swim chamber zones were monitored every minute over 20 h using a HOBOware U30 data-logger with 12 bit temperature sensors (Onset Computer Corporation) which displayed zone temperatures of 16°C (15.92 ± 0.15), 19°C (19.19 ± 0.14, 18.70 ± 0.10) 21°C (21.36 ± 0.23, 20.86 ± 0.12), 23°C (23.39 ± 0.12, 23.12 ± 0.18) and 26°C (26.00 ± 0.10). ATPT design and validation can be found in Figure 2.1.

2.3 Tracking and quantification of goldfish behaviours

Tracking of goldfish movement was accomplished by installation of infrared lighting and camera systems directly above the ATPT which gave a bird's eye view of the apparatus. This allowed continuous digital video taping of fish movement within the apparatus in both simulated day and night cycles over 72 h in zymosan based experiments, or 14 days in *Aeromonas* infections. Videos were loaded into Ethovision XT 11 (Noldus, Netherlands). An 'arena' was drawn over the continuous swim chamber portion of the ATPT and segmented into 8 'zones' which contained respective temperatures of 16°C, 19°C, 21°C, 23°C or 26°C. Ethovision detects fish based on size and contrast and uses a 'dynamic subtraction' method that normalizes background images to improve detection of moving animals. This program uses detection to autonomously designate coordinates to targets within the APT, allowing for the quantification of behaviours based on placement and movement of each individual fish with extremely high temporal resolution limited only by video frame-rates. Following automated tracking of each video, tracking of fish was verified for accuracy and edited by hand to correct for any incorrect detections such as light reflection off the water surface or

condensation. Behavioural tracks also required manual tracking and editing during sudden changes in contrast such as switching of light cycle conditions or camera flaring, which is common in infrared camera systems. Once tracks had been thoroughly edited, data analyses allowed compilation of temperature zone, velocity and transition between zones exhibited by each fish on a per second basis, generating approximately 86000 data-points per day of analysis. These raw data sheets were then exported to Microsoft Excel and each trial was compiled before reanalysis to find mean hourly temperature preference, velocity and total hourly zone transitions. Ethovision arena design and detection can be found in Figure 2.2.

2.4 Culture Medias

2.4.1 Fish serum

Common carp (*Cyprinus carpio*) held in the University of Alberta Aquatics Facility were anesthetized in a 50 mg/L TMS solution before being bled from the caudal vein. Blood was pooled, coagulated overnight and spun at 1000 x g at 4°C for 30 minutes. Serum was collected and heat inactivated in a 56°C water-bath for 30 minutes, then filter sterilized through 0.22 micron filters. Aliquots were frozen at -20°C and thawed for use in culturing primary goldfish leukocytes during functional *ex vivo* assays.

2.4.2 Modified Goldfish Leibovitz-15 media (MGFL-15)

Modified goldfish Lebovitz-15 (MGFL-15) medium used to culture primary isolated cells and has been previously described by the Belosevic lab (467). Briefly, one packet of Lebovitz-15 (Gibco) and Dulbeco's Eagle medium (Gibco) are dissolved in two liters of water. MEM amino acid, non-essential amino acid and vitamin solutions acquired from Gibco were added along with bovine insulin (Sigma-Aldrich), sodium pyruvate, various salts and lab-made nucleic acid precursor and 10x Hank's balanced salt solutions. Finalized solutions were adjusted to a pH of 7.3-7.4, filter sterilized and stored at 4°C. Complete MGFL-15

(cMGFL) contains 10% Carp serum. Tables containing compositions of MGFL-15, nucleic acid precursor and 10x HBSS are listed in Table 2.1, 2.2 and 2.3 respectively.

2.4.3 Hank's Balanced Salt Solution ($HBSS^{+/+}$)

Hank's balanced salt solution ($HBSS^{Ca2+Mg2+}$) was used to culture primary isolated cells destined for flow cytometric analysis due to its lack of fluorescent phenol red, and has previously been shown by Havixbeck *et al* to retain high viability of primary isolated goldfish neutrophils (244). Dry components were purchased from Sigma-Aldrich and stored at room temperature before $HBSS^{Ca2+Mg2+}$was created using MilliQ treated water. Final HBSS solutions were adjusted to a pH of 7.3-7.4, filter sterilized and stored at 4°C before usage. Complete HBSS (cHBSS) contained 10% Carp Serum. Composition of $HBSS^{+/+}$ is listed in Table 2.4.

2.5 Isolation of peritoneal leukocytes

2.5.1 Intraperitoneal injections

Goldfish of roughly 4-5" in length and one year of age were anesthetized in a TMS solution and placed on bench coat. Fish were injected using 23½ gauge needles loaded with 100 µL of 50 mg/mL zymosan solution isolated from *Saccharomyces cerevisiae (*Alfa Aesar) or 1x $PBS^{-/-}$ (phosphate buffered saline). Injections were performed through the pocket of soft tissue behind the pectoral fin, and injected into the body cavity at a 45° angle relative the body and pointed in a slightly posterior direction. Goldfish were then returned to regular 16°C water containing air bubblers to recover from anesthesia.

2.5.2 Peritoneal Lavage

Goldfish were anesthetized in TMS and sacrificed via cervical dislocation prior to peritoneal lavages. Terminated fish were placed on bench coat, and a flap was cut into the peritoneum around the outline of the pectoral fin to a final size of approximately 1 cm^2, though this size varied slightly with both body and fin size. Ten milliliter syringes loaded

with ice cold 1x $PBS^{-/-}$ and tipped with 18 gauge needles with manually rounded tips were used to flush the peritoneum. Two to three scales were removed just behind the head and above the midline where needles were inserted in an anterior-posterior direction into the peritoneum before PBS was delivered with constant pressure. Peritoneal lavage liquid was collected into 50mL conical tubes through the window cut around the pectoral fin and was used directly for cell counts and DHR analysis, or was spun at 335 *x g* and resuspended in respective medias for other *ex* vivo assays.

2.5.3 Quantification of peritoneal leukocytes

Concentration of peritoneal leukocytes in lavage fluid was enumerated using a hemocytometer (Fisher) and light microscopy on a Nikon Eclipse TS100 inverted light microscope. Cell concentration was multiplied by the total volume to yield total leukocyte counts in each lavage. Cell subpopulations were determined by cytohistochemical staining.

2.5.3.1 Quantification of leukocyte subpopulations

Peritoneal lavage fluid was spun at 335 *x g* and resuspended at a concentration of 1 x 10^6 cells/mL, and 100µL was loaded into a spin column and spun onto glass slides at 41 *x g* using a Cytospin 4 cytocentrifuge (Shandon, Thermo). Cells were fixed to slides using a cold glutaraldehyde fixative solution (4% in borate buffer with 25% acetone) for one minute with constant agitation. Fixed cells were rinsed with distilled water and slides were flooded with Sudan black B staining solution. Slides were stained for 5 minutes with intermittent agitation, rinsed and counter-stained for another 5 minutes with Hematoxylin solution Gill No. 3 for 5 minutes followed by final rinse with tap water. All staining reagents were purchased from Sigma-Aldrich. Slides were visualized under oil immersion at 1000x magnification on a Leica DM1000 confocal microscope. Neutrophil, macrophage-monocyte and lymphocyte subpopulations were counted based on morphology and Sudan black positivity as previously described (468). Percentages of population for each sample was

multiplied into total lavage leukocyte numbers to back-calculate total numbers of neutrophils, monocytes-macrophages or lymphocytes per lavage.

2.6 Bacteria

2.6.1 *GFP Escherichia coli DH5α*

DH5α *Escherichia coli* expressing green fluorescent protein was stored at -80°C in a concentrated glycerol stock. Frozen stocks were inoculated into sterile Luria-Bertani broth (BD Biosciences) and grown at 37°C in a shaker overnight before experimental use. The concentration of bacterial cultures was determined before each experiment by optical density using 600nm light and calculated against a growth curve constructed from O.D. 600 and colony formation on LB plates (Figure 2.3). Stocks were spun at 13000 *x g* for 2 minutes and washed with 1x $PBS^{-/-}$ twice prior to experimental use.

2.6.2 *Aeromonas veronii*

Aeromonas veronii was previously isolated and identified by Dr. Aja Rieger and Dr. Jeff Havixbeck from natural lesions found on goldfish held in the University of Alberta's Aquatics facility (469). Colony PCR and sequencing identified this strain as *Aeromonas veronii* biovar sobria, of which a clonal stock was generated and stored at -80°C in a glycerol solution. Frozen stocks were inoculated into sterile trypticase soy broth (BD Biosciences) and grown fresh overnight at room temperature on a culture spinner before each experiment. Bacterial concentration was quantified using spectrophotometry and an O.D. 600nm growth curve (Figure 2.4). *Aeromonas* cultures were spun at 13 000 *x g* for two minutes and washed with 1x PBS prior to experimental use in *ex vivo* killing assays or inducing live *Aeromonas* infections in goldfish. *Aeromonas* cultures were also heat killed at 80°C for 60 minutes and washed twice with 1x$PBS^{-/-}$ as previously described (469). Heat killed bacteria were then stained in 1μM propidium iodide (ThermoFisher) for 60 minutes and washed twice for use in phagocytosis assays.

2.7 Functional leukocyte bio-assays

2.7.1 Generation of reactive oxygen species

This assay was performed as previously described (242,243). Peritoneal lavage fluid was transferred to 5mL round bottom FACS tubes (BD Falcon) to a volume of 200µL. Dihydrorhodamine (DHR, ThermoFisher, Molecular Probes) was added to the cells to a final concentration of 10µM and incubated for 5 minutes to allow cellular incorporation prior to addition of PMA (Phorbol 12-myristate 13-acetate, Sigma-Aldrich) to a final concentration of 1.6µM, which has been previously shown to induce DHR primed fish leukocytes to produce ROS (242). After 30 minutes of incubation at 16°C, samples were analyzed for fluorescence exhibited by oxidized DHR by conventional flow cytometry on a FACS Canto II platform (BD Biosciences). Multiple samples were staggered by 5 minutes to account for the short lifespan of rhodamine fluorescence.

2.7.2 Generation of nitric oxide

This assay was performed with modification as described in goldfish somatotropes (470). Following isolation via peritoneal lavage, cells were spun at 335 *x g* and resuspended in cHBSS$^{Ca2+Mg2+}$ and 4-amino-5methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes, Life Technologies) was added to a concentration of 1µM. DAF-FM DA is a cell membrane-permeant reagent that reacts with nitric oxide to produce the stable, membrane-impermeant and fluorescent benzotriazole DAF-FM. After 1 hour of incubation at 16°C, samples were analyzed for fluorescence using conventional flow cytometry on a BD FACS Canto II.

2.7.3 Phagocytosis of *E. coli*

Peritoneal leukocytes were isolated at the peak of cellular infiltration (16 hpi) were spun at *335 x g* and resuspended in cHBSS$^{Ca2+Mg2+}$. Two million cells were seeded into 5mL round bottom tubes (BD Falcon) in 200µL of media, and 5 million live GFP expressing *E. coli*

were added. Replicate tubes were incubated at 16°C or 26°C for 15, 30 or 60 minutes before fixation in 1% formaldehyde. After 20 minutes of fixation at 4°C, cells were spun at 424 *x g* to account for cell shrinkage due to fixation and resuspended in 100µL 1x PBS with 5µL of Nucblue Live ReadyProbes reagent (Hoescht33342 solution, Molecular Probes, Life Technologies) and incubated for a further 20 minutes to allow staining. Cell images were then captured on the Imagestream MkII imaging flow cytometer platform (Amnis, EMD Millipore) and analyzed for *E. coli* internalization using IDEAS 6 software as previously described (242).

2.7.4 Assessment of leukocyte ROS and NO response to *Aeromonas veronii* internalization

Peritoneal leukocytes isolated at 16 hpi were spun at *335 x g* and resuspended in cHBSS$^{Ca2+Mg2+}$. These cells were seeded into 5mL round bottom tubes (BD Falcon) at a density of 2x10^6 cells in 200 µL of media along with 2.5:1 heat-killed *Aeromonas veronii* stained with propidium iodide. DAF-FM DA (ThermoFisher) was added at this time at a concentration of 1 µM to allow for cellular incorporation. CellROX deep red (ThermoFisher) is a cell permeant non-fluorescent dye that becomes a stable fluorophore upon direct oxidation by reactive oxygen species, and was added to a final concentration of 6.25 µM 30 minutes into incubation. After a total of 60 minutes of co-incubation at 16°C, samples were fixed in 1% formaldehyde at 4°C for 20 minutes. Samples were then spun at 424 *x g* and resuspended in 100µL 1x PBS with 5 µL of Nucblue Live ReadyProbes reagent (Hoescht33342 solution, Molecular Probes, Life Technologies). Following another 20 minutes at 4°C to allow staining, cell images were captured on an Imagestream Mk II imaging flow cytometer and analyzed for *A. veronii* internalization as previously described (242). Leukocytes with internalized bacteria were then analyzed for the generation of ROS and NO by positive staining of DAF-FM or CellROX deep red respectively.

2.7.5 *Ex vivo* bacterial killing assay

Goldfish peritoneal leukocytes were isolated at 12 or 16 hpi, washed with 1x $PBS^{-/-}$ at 335 *x g* and resuspended in cMGFL-15 to a density of $2x10^6$ cells in 200 µL. *Aeromonas veronii* was added to a final target-effector ratio of 2.5:1. Tubes were incubated at 16°C or 26°C and 10 µL aliquots were removed at 0, 15, 30 or 60 minutes and lysed in 90 µL of MilliQ water for 60 seconds to release internalized bacteria that had yet to be functionally killed by intracellular mechanisms. Osmolality was then readjusted with 10 µL 10x $PBS^{-/-}$ after which samples were serial diluted in 1x PBS $^{-/-}$ and dilutions were plated onto TSA agar plates. These plates were grown overnight at room temperature and assessed for CFUs, and thus live bacteria cells.

2.8 Live *Aeromonas* infection model

2.8.1 Induced furunculation

The model of induced *Aeromonas* furunculation was performed as previously described (469). Goldfish were anesthetized in a TMS solution and placed on dry bench coat. A 4x4 patch of scales centered on the mid-line was removed from each fish and a sterile needle was used to score the underlying skin. A sterile swab was used to inoculate the wound with a concentrated *Aeromonas veronii* culture which was allowed to absorb into the wound for 7 seconds before being revived in regular 16°C water containing an air bubbler. Fish were monitored for furuncle formation at 1, 2, 4, 7, 10 and 14 days, at which point fish were anesthetized and sacrificed. Infected furuncles were assessed for *Aeromonas* load, removed from the fish and flash frozen in liquid nitrogen for use in molecular analysis.

2.8.2 Detection of bacterial load

At respective days-post-infection, fish were anesthetized in TMS solution and sacrificed. A sterile swab was rolled into the furuncle, coated with local mucous, and was then used to streak the entire surface of a TSA agar plate. These plates were incubated

overnight at room temperature and assessed for CFUs as a measure of bacterial shedding. Dr. Jeff Havixbeck has previously shown that swabbing of uninfected fish, or even on scales on the contralateral side on an infected fish yields insignificant numbers of CFUs (469).

2.9 Quantitative PCR

2.9.1 RNA extraction

2.9.2 Peritoneal Cells

Goldfish leukocytes were collected via peritoneal lavage and lavage fluid was spun at 335 *x g* for 10 minutes. Supernatants were decanted well and remaining cell pellets were frozen at -80°C until RNA extractions were performed on ice using an RNeasy kit (Qiagen) according to manufacturer's protocol. Briefly, leukocytes were lightly thawed and lysed in 500 µL RLT lysis buffer, at which point 500 µL of 70% ethanol was added. Solutions were mixed and loaded into spin columns and centrifuged at 10 000 *x g* for 20 seconds. Columns were then washed and spun once with RW1 Buffer and twice with RPE Buffer followed by a 2 minute spin to remove excess liquid. Spin columns were placed into labelled Eppendorf tubes and 30 µL of Ambion nuclease free water was added prior to a final 1 minute spin in order to elute RNA from the column. The concentrations of collected RNA samples were measured by absorbance of 260 nm light using a Nanodrop (Thermo) microvolume spectrophotometer. Contamination of proteins and phenolic compounds was also assessed using absorbance of 280 nm and 230 nm light respectively. Samples were stored at -80°C until being used to synthesize cDNA.

2.9.3 Tissues

Goldfish were anesthetized and sacrificed prior to dissection of brain or infected furuncles. Tissues were flash frozen in liquid nitrogen as quickly as possible post-termination and were stored at -80°C until RNA extraction. Frozen tissues were homogenized in 1 mL of TRIzol Reagent (Invitrogen, ThermoFisher) using a PRO 200 double insulated blade disruption

homogenizer. One milliliter of each homogenized sample was transferred to respective Eppendorf tubes along with 100 µL of chloroform. Tubes were then vortexed, left to sit on ice for 5 minutes and re-vortexed prior to being spun at 12 000 *x g* for 30 minutes at 4°C. The aqueous layer was then transferred to a new labelled tube with care to not disrupt other layers. One volume of isopropanol was added to each tube and mixed by inversion before being stored at -20°C overnight to allow RNA precipitation. Tubes were then spun at 12 000 *x g* at 4°C at which point supernatants were decanted well and remaining RNA pellets were washed with 75% ethanol. Following a final 30 minute spin, supernatants were decanted, and pellets were dried with open tube caps at room temperature for approximately 5 minutes to allow ethanol evaporation. Samples were then resuspended in 50 µL of nuclease free water (Ambion) and quantified as outlined above in Section 2.9.2.

2.9.4 cDNA synthesis

cDNA was synthesized from collected RNA samples using SMARTScribe reverse transcriptase (Clontech, Takara Bio Inc., Japan) and poly-dT according to manufacturer's instructions. RNA samples were standardized by weight (200 ng for cells, 2 µg for tissues) and first strand of cDNA was synthesized by cycling to 72°C for 3 minutes and then 42°C for 60 minutes. The second strand was synthesized using a ratio of 100:1 Taq to Pfu DNA polymerases, and cycled to 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 15 minutes. cDNA samples were either used immediately or stored at -20°C for qPCR analysis.

2.9.5 Quantitative PCR conditions

Quantitative PCR of cDNA samples generated from goldfish tissue or isolated leukocytes was completed using a 7500 Fast Real Time PCR machine (Applied Biosystems) and SYBR green reagent mix prepared by Molecular Biology Services Unit staff at the University of Alberta. cDNA samples loaded with respective primers and SYBR green mix were heated to 95°C for 10 minutes and then cycled 40 times between 60°C for 60 seconds and 95°C for 15 seconds. Raw data was analyzed using Applied Biosciences' accompanying 7500 Fast

software using the average of 3 technical replicates for each sample. Elongation factor 1α was used as an endogenous control and results are reported as relative quotient (RQ) of gene expression in control 0 hour or uninfected fish.

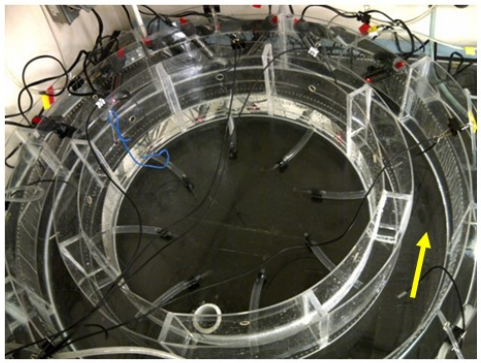
2.9.6 Primers

Primers used in the generation of cDNA and in qPCR are listed in Tables 2.6 and 2.7 respectively. Primers used in qPCR analysis were previously validated in goldfish models (244,335,471–473).

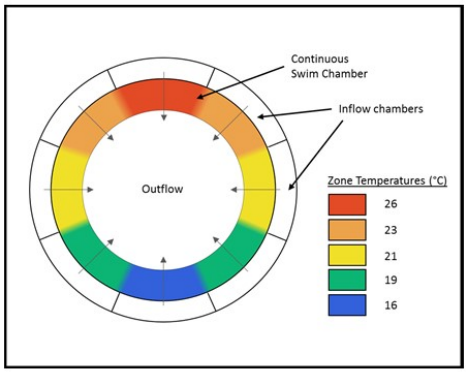
2.10 Statistics

All t-tests and one or two way ANOVAs using Sidak's or Tukey's post-hoc tests were calculated using Prism 7.02 (Graphpad) and are notated as $F_{D,n}$ = F statistic, where D = the degrees of freedom, and n = the number of replicates in the test. Mean values and correlations for behavioural data were calculated in Excel (Microsoft). R was used to calculate multivariate statistics including principle component analysis (standard R package) and permutational multivariate analysis of variance using distance matrices (vegan package)(474).

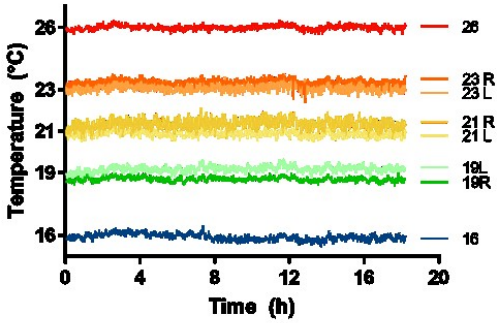
A



B



C



D

Zone	Temp ± SD (°C)
26	**26.00 ± 0.10**
23 R	23.39 ± 0.12
23 L	23.12 ± 0.18
21 R	**21.36 ± 0.23**
21 L	**20.86 ± 0.12**
19 R	19.19 ± 0.14
19 L	18.70 ± 0.10
16	**15.92 ± 0.15**

E

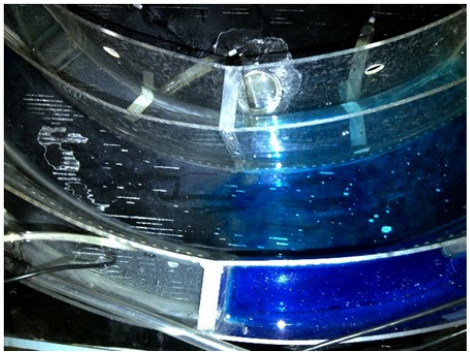


Figure 2.1 ATPT design and validation. A) The annular temperature preference tank is designed to generate a continuous ring shaped swim chamber (yellow arrow) a containing range of temperatures without using solid barriers as **B)** illustrated by an aerial view schematic. **C)** When measured with automated temperature loggers, the apparatus is capable of generating stable temperature zones maintained by fluid dynamics alone. **D)** The mean temperature was measured over 20 hours for each zone. **E)** Flow is mediated from input chambers, across the swim chamber towards drainage at the center of the apparatus which generates distinct zones as validated with a fluid dye test.

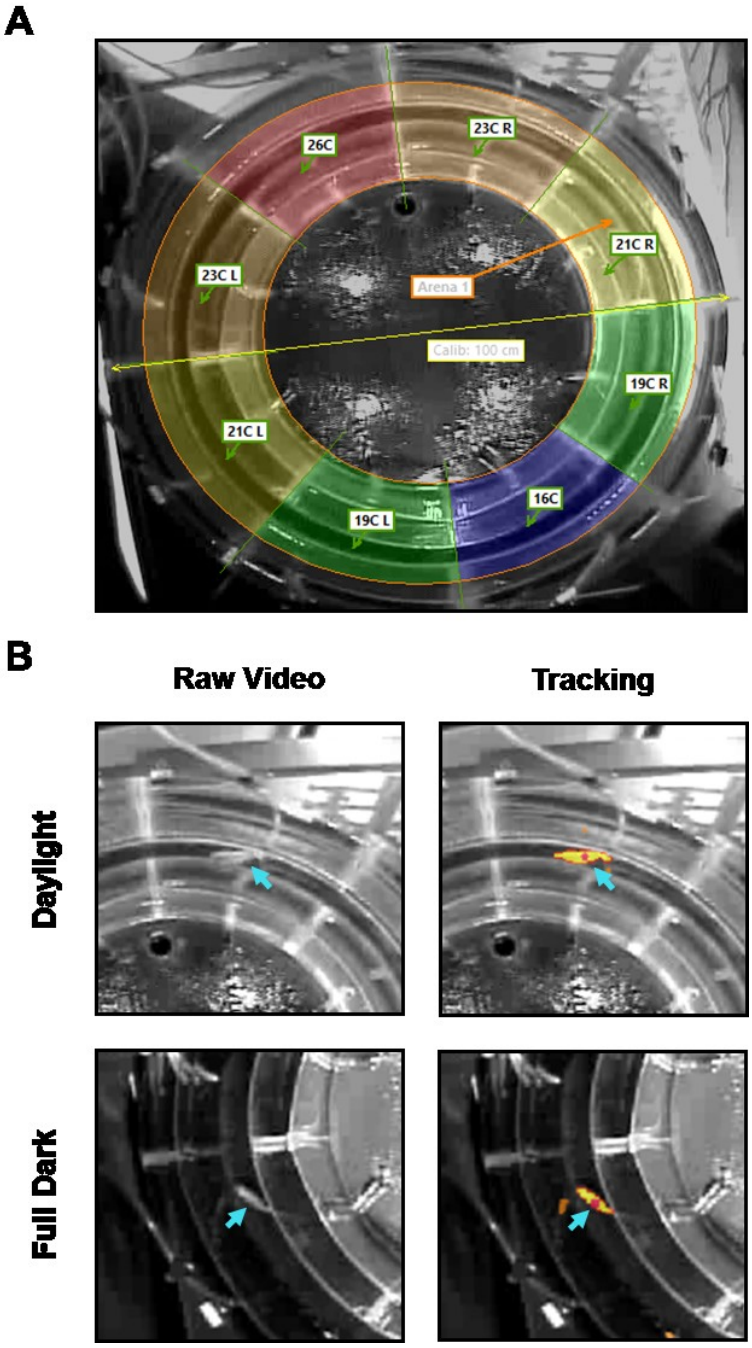


Figure 2.2 Tracking of goldfish behaviour. Infrared video of goldfish movement within the ATPT was loaded into Ethovision XT 11 and **A)** an 'arena' was drawn around the continuous swim chamber. This arena was divided into 8 segments of 16, 19, 21, 23 and 26°C depending on zone temperature The arena was calibrated to ATPT size to ensure accurate behavioural outputs. **B)** A dynamic subtraction technique was used to detect fish within raw videos (blue arrows). The use of infrared lighting and camera systems allowed for the continuous scanning of fish during simulated daylight and night (Full Dark) conditions.

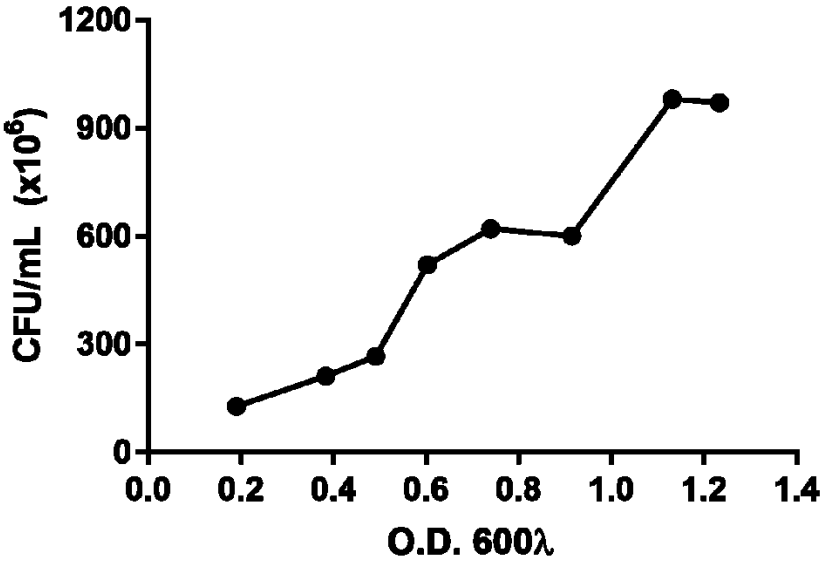


Figure 2.3 GFP *Escherichia coli* growth curve. GFP-expressing *E coli* were growth from frozen stocks in liquid Luria-Bertani broth at 37°C overnight. This stock was then diluted in fresh LB broth and incubated in 37°C. Each hour bacterial stocks were assessed for optical density using 600nm light, serial diluted and plated onto LB agar. Plates were incubated overnight at 37°C and CFUs were enumerated.

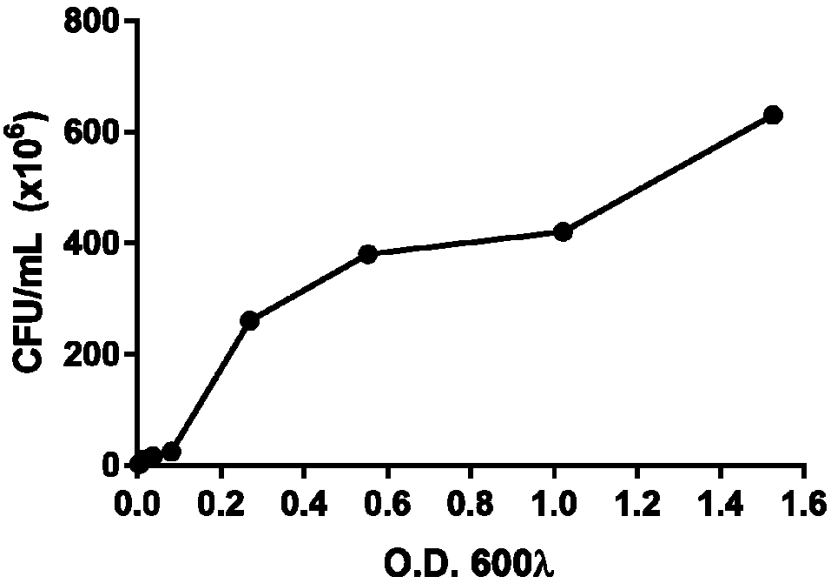


Figure 2.4 *Aeromonas veronii* growth curve. *Aeromonas* veronii was grown from frozen stocks in trypticase soy broth at room temperature. Optical density of the culture to 600nm light was determined by spectrophotometry each hour and an aliquot of culture was serial diluted on trypticase soy agar plates. Plates were incubated at room temperature overnight and CFUs were enumerated.

Table 2.1 Composition of Modified Goldish Leibovitz-15 media

Component	Quantity
KH_2PO_4	0.69 g
K_2HPO_4	0.57 g
NaOH	0.75 g
$NaHCO_3$	0.34 g
HEPES	7.00 g
L-glutamine	0.584 g
Bovine Insulin	0.01 g
GFL-15*	1L
10x Hank's Balanced Salt Solution	80 mL
Nucleic acid precursor solution	20 mL
MEM amino acid solution	25 mL
MEM non-essential amino acid solution	25 mL
Sodium pyruvate solution	25 mL
MEM vitamin solution	20 mL
β-mercapto-ethanol	7 µL
MilliQ water	fill to 2L

*GFL consists of Leibovitz-15 and Dulbeco's Modified Eagle Medium dissolved in 2 L of MilliQ water

Table 2.2 Composition of nucleic acid precursor solution

Component	Quantity
MilliQ water	100 mL
Adenosine	0.067 g
Cytidine	0.061 g
Hypoxanthine	0.034 g
Thymidine	0.061 g
Uridine	0.061 g

Table 2.3 Composition of 10x Hank's Balanced Salt Solution

Component	Quantity
MilliQ water	fill to 500mL
NaCl	40.00 g
$NaHPO_4 \bullet 7H_2O$	2.00 g
KCl	2.00 g
KH_2PO_4	0.30 g
D-glucose	0.05 g
Phenol red	0.05

*This stock is not used to generate 1x $HBSS^{+/+}$ due to the presence of phenol red

Table 2.4 Composition of calcium and magnesium supplemented 1x Hank's Balanced Salt Solution

Component	Quantity
MilliQ water	fill to 1 L
NaCl	8.0 g
$NaHPO_4 \bullet 7H_2O$	0.048 g
KCl	0.40 g
KH2PO4	0.06 g
D-glucose	1.0 g
$MgCl_2 \bullet 6H2O$	0.1 g
$MgSO_4 \bullet 7H2O$	0.1 g

Table 2.5 Composition of 10x Phosphate Buffered Saline

Component	Quantity
MilliQ water	fill to 1 L
NaCl	8.0 g
$NaHPO_4 \bullet 7H_2O$	21.6 g
KCl	2.0 g
KH_2PO_4	2.0 g

*This stock is diluted with filter sterilized MilliQ water to generate 1x $PBS^{-/-}$

Table 2.6 Primers used in cDNA synthesis

Primer	Sequence (5' → 3')
5' oligo	AAG CAG TGG TAT CAA CGC AGA GTA CG
3' CDS poly T	AAG CAG TGG TAT CAA CGC AGA GTA TT
5' PCR	AAG CAG TGG TAT CAA CGC AGA GT

Table 2.7 Quantitative PCR primers

Primer	Sequence (5' → 3')
IL-1β1 forward	GCG CTG CTC AAC TTC ATC TTG
IL-1β1 reverse	GTG ACA CAT TAA GCG GCT TCA C
TNFα forward	TCA TTC CTT ACG ACG GCA TTT
TNFα reverse	CAG TCA CGT CAG CCT TGC AG
TGF-β forward	GTA CAC TAC GGC GGA GGA TTG
TGF-β reverse	CGC TTC GAT TCG CTT TCT CT
IL-10 forward	CAA GGA GCT CCG TTC TGC AT
IL-10 reverse	TCG AGT AAT GGT GCC AAG TCA TCA
CXCL-8 forward	CTG AGA GTC GAC GCA TTG GAA
CXCL-8 reverse	TGG TGT CTT TAC AGT GTG AGT TTG G
VEG-F forward	ATG AGA ACC ACA CAG GAC GGG ATG TA
VEG-F reverse	CGA GAG CTG CTG GTG CTG GTA GAC ATC ATT
iNOS-A forward	TTG GTA CAT GGG CAC TGA GAT T
iNOS-A reverse	CCA ACC CGC TCA AGA ACA TT
EF-1α forward	CCG TTG AGA TGC ACC ATG AGT
EF-1α reverse	TTG ACA GAC ACG TTC TTC ACG TT

Chapter 3: Behavioural fever is a potent regulator of functional innate immune responses

3.1 Introduction

Fever has long been recognized as a response to infection, and is commonly thought to be associated with pathology and mortality in medical settings. This paradigm is slowly changing, as several clinical studies have shown that the interruption of fever actually increases mortality rates in critically ill patients, suggesting that fever plays an important role in pathogen defence. Research into fever has been hampered by the inability to prevent febrile induction in experimental settings due to physiological stress generated by externally cooling the body, or by off-target effects of pharmaceutically blocking fever using non-steroidal anti-inflammatory drugs (1,3,101). In the 1970s, it was noted that cold-blooded animals would also warm themselves during infection to generate a 'behavioural fever', and that this increase in body temperature drastically improved pathogen clearance and survival during infection (131,475). Upon the identification of a vast range of species that could elicit these types of responses (see *Figure 1.1* for phylogenetic distribution), behavioural fever in ectotherms was identified as a comparative model to study the effects of fever without the interference of intrinsic thermoregulatory mechanisms. Likely due to a lack of reagents in ectothermic model animals, interest into behavioural fever as a comparative model dropped until more recently with the improvement of molecular techniques at the turn of the century. While several studies have displayed upregulation of inflammatory or antimicrobial genes caused by behavioural fever, none have assessed the effect of this response on the functional inflammatory reactions that might play a role in promoting improved pathogen clearance and thus survival (132–134,142). To address this gap of knowledge in the field, the goal of my project was to use goldfish as a model to assess the effect of behavioural

fever and thermoregulation on the kinetics and functionality inflammatory leukocyte responses.

This chapter describes my use of a zymosan-induced peritonitis model to [1] quantify febrile behaviours in goldfish with unprecedented temporal resolution, [2] assess the effects of these behaviours on local leukocyte responses within the peritoneum to identify immuno-modulatory effects induced by behavioural fever and [3] assess the effect of this temperature range during infection using a previously developed dermal *Aeromonas veronii* induced furunculation model that mimics the natural infections caused by this pathogen in teleost fish (469).

Herein, my research focuses on the ability to thermoregulate to impact inflammatory responses and further, the outcome of infections. I define the use of 'behavioural fever' as a temporal increase in behavioural thermopreference induced by microbial or immune sources. While this term has historically been used throughout the literature to describe any temperature related improvement of pathogen clearance, it is possible that basal thermoregulation to a species homeostatic temperature and not a true homologous pathogen dependant fever can impact inflammation or pathogens to alter infection outcomes. Therefore, I make the distinction of 'behavioural or ectothermic fever' as a specific subset of behavioural thermoregulation.

3.2 Zymosan peritonitis generates febrile behaviours in goldfish characterised by altered thermal preference and lethargy

While it is known that a wide range of ectothermic animals will seek warmer temperatures under infection, little is known about febrile kinetics like how soon after

infection it begins or the timeframe that it persists, especially on an individual basis. To study behavioural fever in our goldfish model, I assessed the ability of intraperitoneal zymosan injection, known to induce fever in traditional models and altered thermal preference in invertebrates, to generate a febrile response (459,476).

There are several types of aquaria commonly used to study thermopreference in aquatic organisms including vertically stratified tanks, horizontal flow through gradients and shuttlebox configurations which contain different temperature waters connected by tunnels (133,134,142). While these apparatus may allow a range of temperatures to be established for animal selection, other factors such as variable depth and currents can play a role in animal positioning within the apparatus (477). Flat edges and physical barriers can have particularly large impact on positioning, as flat surfaces can be perceived as cover by fish which may not leave cover to search for other water temperatures. In addition, many of these studies use multiple animals at once, and just measure density of animals in different regions through time. Fish can have complex social interactions spanning from schooling and grouping to territorial avoidance which may skew accurate readouts of temperature alone (478,479). In addition, most studies track positioning ever 5-60 minutes over a time course, which allows for only the calculation of a population temperature preference that may be skewed to for the variety of reasons outlined above, and with poor temporal resolution which eliminates the ability to measure other fever associated behaviours.

Of the many methods used to quantify temperature preference in fish, I chose the annular temperature preference tank (ATPT) first designed by Myrick *et al* to specifically study thermopreference in aquatic organisms (466). The ATPT was designed to generate an arena of various temperature water using fluid dynamics instead of physical barriers allowing the animal to freely choose its own temperature independent of variable lighting, perceived cover, depth or current that may impact positional behaviour, and was modified to establish a range of 16-26°C (**Figure 2.1**). When coupled to an individual tracking

system with high temporal resolution, this system removes normalization of group behaviour (e.g. due to schooling) which allows for greater analytical robustness than seen in previous behavioural fever studies. When moved between either end of this temperature range, goldfish internal body temperature equalizes within 2.5 minutes, indicating a relatively short period of time is required to alter body temperature by behaviour within the ATPT **(Figure 3.1).** Behavioural analysis revealed a distinct timeframe from 0-20 hours post-injection (hpi) with an overall trend of ~1°C increase in mean thermopreference of zymosan injected fish when compared to PBS injected control. This is clearly displayed at 4-8 hpi with a mean thermal preference of 23.77 ± 0.26°C for zymosan injected fish and 22.43 ± 0.38°C for mock injection controls (**Figure 3.2A)**. Outside of this 0-20 hpi window, temperature preference in stimulated fish fell around 23°C with large fluctuations both temporally and between individual fish, similar to saline control. Utilizing per second tracking resolution, other behaviours were assessed that compliment increased thermal preference as a febrile behaviour and allow me to better define the febrile response in goldfish. A period of minimal mean velocity was seen to overlap the window of high temperature preference in zymosan fish, while mock injected fish mobility remained highly variable during the same timeframe (**Figure 3.2A**). I also analysed temperature seeking behaviour by quantifying the number of transitions each fish made between temperature zones. During the same ~0-20 hpi window, zymosan treated fish rarely switched temperature zones compared to several hundred transitions per hour for mock injection controls (**Figure 3.2A**). These results indicated that overall, zymosan stimulation drives an increase in body temperature, but other 'sickness behaviours' such as lethargy, fatigue and malaise define the febrile process in teleosts.

A series of pro-inflammatory factors like IL-1β, IL-6 and prostaglandin E2 are known to trigger fever across mammals and various ectotherms by inducing the expression of similar factors in intracranial endothelial cells near the hypothalamus to functionally cross

the blood-brain barrier and act on thermoregulatory neurons to increase temperature set point (4,66,76,480). Hence, I assessed IL-1β expression in the brain as a possible conserved mechanism to initiate febrile responses in teleosts. We found that intraperitoneal zymosan injection causes a small increase in IL-1β expression in brain tissues by 8 hpi, though fish that were able to thermoregulate displayed increased expression by 4 hpi (**Figure 3.3**). Though two way ANOVA analysis revealed a significant effect of zymosan on the expression of IL-1β ($F_{1,64}$, p=0.005*)*, post hoc analysis revealed no significant difference between pairs. Regardless, this effect is likely to be biologically relevant due to the relatively small few number of meningeal macrophages producing IL-1β in proximity to thermoregulatory neurons within the hypothalamus. Due to this sequestered expression, excision of the hypothalamus and direct analysis of IL-1β may reveal larger, statistically significant changes in expression. The consistency of these symptoms across ectothermic and endothermic fever, as well as their immunological and neural triggers, point to the establishment of a multi-factorial febrile response prior to the divergence of ray-finned fishes and mammals (common ancestor ~ 400 MYA) (4,481,482).

To analyze the data in the context of functional immune effects in a statistical manner, I segmented the behavioural data to look for correlation between behaviours observed and acute inflammatory kinetics previously seen using a zymosan peritonitis mode in goldfish (243,244). I focused on five distinct periods of inflammatory responses: early detection phase (0-4 hpi), initiation of cell recruitment to the site (4-8 hpi), upregulation of leukocyte recruitment and reactive oxygen species (ROS) to peak inflammatory levels (8-20 hpi), early resolving phase where leukocytes begin to leave the site and ROS production declines (20-48 hpi) and late resolving phase (48-72 h) where leukocyte numbers and ROS return to basal levels (**Figure 3.2 B**). Zymosan stimulated fish were seen to show trends of roughly 1°C increased thermopreference through the first three pro-inflammatory phases up to 20 hpi compared to saline controls. Zymosan injected fish chose a mean temperature of 24.13 ± 0.40°C compared to 23.49 ± 0.45°C in controls, though due to large variance

generated in behavioural trace data, these differences were not statistically significant (**Figure 3.2 B**). In sharp contrast, movement remained lower in zymosan injected fish than saline injected controls until around 48-60 hpi, with both mean velocity (PBS 1.98 ± 0.58cm/s, Zym 0.48 ± 0.16 cm/s, p = 0.008) and mean zone transitions (PBS 112.75 ± 42.28, Zym 18.02 ± 7.64, p = 0.003) become statistically lower during the main 8-20 hpi pro-inflammatory window (**Figure 3.2B**).

All hourly values for individual animals from both treatment and control groups were pooled for multivariate analysis (**Figure 3.2C**). While both groups showed periods of high thermopreference (24-26°C), both velocity and temperature seeking behaviour of zymosan fish remained exclusively low during these periods while control fish exhibited a wide range of movement profiles. Correlative analysis supports little relationship between temperature preference and velocity for PBS controls (R=0.02 ± 0.14), but a negative correlation in zymosan injected fish (R=-0.35 ± 0.14, p = *0.06*) (**Figure 3.4**) indicating that periods of high temperature preference in fish are accompanied by lethargic behaviours, a well-established symptom of mammalian fever. Although these results are not statistically significant to a 95% confidence interval, a difference is likely due to the inherent noise involved in behavioural data when paired to the relatively small sample size. Principle component analysis was used to calculate the relative impact of each factor or readout on the total dataset variance. This is done by calculating the relative effect of each factor on principle components that gauge the greatest range of variance in decreasing order, with principle component (PC) 1 explaining the most variance, then PC2, and so on. This analysis revealed that large amounts of variance within the first two principle components were due to the type of injection (zymosan vs control) and individuality of replicate fish, with loadings of PC1: -0.42, PC2: -0.57 for treatment and PC1:-0.51, PC2:-0.43 for fish ID respectively. This indicates that the type of injection and individual behavioural phenotype of each fish had a high impact on the overall variance within the dataset **(Figure 3.5)**. Regardless of

high variance within the data, a permutational multivariate analysis of variance using distance matrices showed statistically significant impacts of fish ID, treatment, time and treatment•time interaction on dependant behavioural read-outs (temperature preference, velocity and temperature zone transitions) to a significance level of $p < 0.001$ (**Table 3.1**). These results indicate that the behavioural readouts of temperature preference and movement are not only significantly impacted by treatment with zymosan, time, and the behavioural phenotype of each fish alone, but also by the interaction of time and treatment. Together, these results define behavioural fever as a multi-factorial biological process in teleosts that shares the thermal preference and lethargy seen in the febrile responses of higher endothermic vertebrates.

3.3 Behavioural fever accelerates the kinetics of cellular infiltration to the inflammatory site but also promotes an early return to homeostatic levels

Others have previously shown that behavioural fever results in enhanced pathogen clearance and host survivability (130,132,460,475), but there is little known about the underlying immunomodulatory mechanisms that govern this effect. To answer this question, I began by looking at leukocyte recruitment to the peritoneum in response to zymosan injection between goldfish given the ability to choose their temperatures within the ATPT here-in referred to as dynamic or T_D fish, and those held in 16°C static temperature aquaria referred to as static or T_{S16} fish. I observed a two-fold increase in leukocytes present in the inflammatory site at 4 and 8 hpi while reaching a statistically significant difference by 12 hpi in T_D fish when compared to T_{S16} controls (**Figure 3.6A**, $p = 0.04$) indicating that increased thermopreference promotes rapid recruitment of leukocytes. Analysis of cell subsets by cytohistochemical staining revealed that this additional influx consisted primarily of

neutrophils that are greatly increased at 4 hpi (T_{S16} = 0.38±0.82 x10^6, T_D = 2.93±0.75 x10^6, *p = 0.07*) and 12 hours (T_{S16} = 2.75±0.78 x10^6, T_D = 6.31±1.28 x10^6, *p = 0.003*) (**Figure 3.6B**). Average values for lymphocyte infiltration in T_D fish were also elevated 2-3 fold over controls from 4 hpi until 16 hpi (**Figure 3.6D**), while monocyte and macrophage levels remained similar to controls until 16 hpi peak of infiltration (**Figure 3.6C**). The peak of cellular infiltration contained similar numbers of monocytes, macrophages and neutrophils present in both T_{S16} and T_D fish. Interestingly, this was followed by a rapid reduction in total peritoneal leukocytes towards basal levels by 20 hpi with *1.95±0.65 x10^6* cells per lavage in T_D fish compared to *8.02±1.79 x10^6* in T_{S16} controls (*p = 0.004*). Neutrophils again appeared to be most affected, with a seven-fold reduction over static temperature controls at 20 hpi (*p = 0.02*). This trend continues through 24 hpi (*p = 0.007*) up to 48 hpi, at which point febrile fish contained nearly half the number of total leukocytes at the inflammatory site but with minimal neutrophilic presence. Although total T_D cell counts decreased compared to T_{S16} at 20-48 hpi, lymphocyte levels in T_D fish remained two fold higher than controls until 48hpi (**Figure 3.6D**). While behavioural fever accelerated leukocyte recruitment kinetics, total infiltration of cells to the site remained similar compared with T_{S16} controls, the exception of lymphocytes which were present to levels over two fold higher in febrile fish (**Figure 3.7).** These results suggests that behavioural fever not only promotes the rapid infiltration of various leukocyte subsets, but also plays a critical role in quickly resolving cellular inflammatory responses and promoting a return to tissue homeostasis.

3.4 Febrile responses prevent high levels of ROS production but promote early NO production in inflammatory leukocytes

To assess whether behavioural fever was modulating not only the rate and composition of infiltrating leukocytes, but also the profile of functional antimicrobial responses they express, I decided to profile the expression of antimicrobial mechanisms expressed by these cell populations. Generation of reactive oxygen species (ROS) is an important and conserved mechanism in both fish and mammals by which cells mediate pathogen clearance and, by extension, host survival (483). In T_{S16} fish, its production closely follows the kinetics of cell infiltration, ramping up by 8-12 hpi and peaking at 16-20 hpi with $69.4 \pm 6.3\%$ and $65.6 \pm 5.6\%$ of total leukocytes positive for ROS production respectively (**Figure 3.6E**). Interestingly, the proportion of ROS producing cells was greatly attenuated under febrile conditions, with T_D fish following static kinetics until 8 hpi with a peak of $20.5 \pm 3.2\%$ ROS+ leukocytes, but anywhere from two to seven fold statistically significant reductions in reactive oxygen production at 12-48 hpi compared to T_S controls. This data suggests that although behavioural thermoregulation appears to accelerate immune cell recruitment to the site, it does not induce the high-level production of a conserved innate killing mechanism as seen in T_{S16} fish. The marked decrease in ROS production may be due to the increased efficiency of early cell infiltration which may reduce the need for high levels of tissue damaging anti-microbial mediators like ROS, possibly allowing the efficient clearance of invaders while reducing collateral damage to host tissues and, by extension, reducing severity and pathology of infection.

Given the decreased capacity to produce ROS seen in these rapidly recruited cells, and the increased pathogen clearance seen in other febrile studies, I hypothesized that behavioural fever might serve to upregulate the production of alternative antimicrobial

mechanisms in responding leukocytes. Thus, I assessed the production of nitric oxide (NO) as a potential alternative killing mechanism. I found that in T_D fish, lowered ROS levels were coupled with accelerated kinetics of NO production to greater overall levels (**Figure 3.6F)**. My results indicated 10-20% leukocyte population producing NO from 8-16 hpi and peaking at 20 hpi (30.40 ± 8.50%), which is significantly higher than T_{S16} controls at the same time point (12.48 ± 3.52, p = 0.007) (**Figure 3.6F**). NO production then decreased through 24 hpi to 10.25 ± 2.00% at 48 hpi in T_D fish, contrasting T_{S16} NO kinetics which start upregulating by 16 hpi to peak at 48hpi (28.18 ± 4.49%), three fold higher than T_D fish at the same time point (p = 0.007). These results indicated that behavioural fever can shift NO responses to peak over 24 h earlier, while being significantly downregulated by peak production of 48 hpi in static temperature controls, thus vastly change the profile of killing mechanisms expressed in the inflammatory site. This shift in NO kinetics was further supported a statistically significant upregulation of inducible nitric oxide synthase (iNOS) expression in T_D fish ($F_{1,32}$=6.11, p=*0.015*) which is required for production of immune NO (**Figure 3.8**) (21).

I also assessed the potential of behavioural fever to affect phagocytic activity in responding leukocytes. I found that while cells obtained from either T_{S16} or T_D fish at peak infiltration (16hpi) internalized GFP-expressing *E.coli* to similar levels when co-incubated at 16°C (**Figure 3.6G**), incubation at 26°C caused a 50% increase in bacterial internalization after 60 min in both groups (T_{S16} p = 0.49, T_D p = 0.52) (**Figure 3.6G**). I concluded that while environmental and thus body temperature increase the kinetics of pathogen internalization, there is no additional activation or priming of cells in febrile fish that increases phagocytic activity *ex vivo*. Regardless, it is likely that increased rates of phagocytosis play a role in febrile clearance, as the *ex vivo* incubation temperature dependent increase in phagocytosis is also $p < 0.001$ likely to occur in *in vivo* leukocyte

populations with fluctuation in body temperature, particularly with increased thermopreference due to behavioural fever.

3.5 Manually increasing static housing temperature does not replicate the immunological effects achieved through active behavioural fever.

While it appeared that behavioural fever was capable of altering cell kinetics and functional activity, I was curious to see if manual temperature manipulation would yield these same observed effects, and if the responses seen were proportionate to increasing body temperature or dependant on a threshold temperature. To this end, fish housed at 16°C were injected with zymosan and immediately moved to static temperature 19, 21, 23 or 26°C aquaria, although due to significant differences in size between animal cohorts, these fish were not compared directly to 16°C T_{S16} or T_D treatments. With few exceptions, our results showed no differences in the kinetics or overall levels of leukocyte infiltration, ROS and NO production between 19°C, 21°C, 23°C and 26°C fixed temperature conditions (**Figure 3.9)**. Further, kinetics for leukocyte infiltration, ROS and NO production were consistent with those shown for 16°C fixed temperature conditions and dramatically different than those achieved through behavioural fever (**Figure 3.9** vs. **Figure 3.6**).

Changes from 16°C to 19, 21, and 23°C yielded no significant changes in total leukocyte infiltration. In contrast, a 10°C increase from basal levels to 26°C was required to elicit a significant increase in total leukocyte infiltration. Unlike the accelerated induction of leukocyte recruitment observed under febrile conditions, a change to 26°C did not cause an early shift in leukocyte recruitment kinetics, nor was static temperature increases capable of accelerating a return of peritoneal leukocytes to homeostatic levels (**Figure 3.9A**).

However, static temperature increases failed to yield any rapid reduction in cell counts from 16 to 20 hpi as seen in T_D, with 26°C actually maintaining significantly higher leukocyte numbers than 19°C and 23°C fish at 20 hpi, with a high proportion of neutrophils remaining at the site (p = 0.02 and 0.01 respectively) (**Figure 3.9A**). Cell sub-populations were also minimally effected, closely following previous 16°C T_{S16} subsets, with the exception of lymphocytes which remain low in 19°C fish, but appear to elevate at 23°C and 26°C with similar kinetics to T_D fish (**Figure 3.9B-D)**.

While increased static temperatures appeared to reduce peak ROS production from 70% in 16°C T_{S16} to 50% at across 19-26°C, the general kinetics remained similar to T_{S16} fish and was upregulated to two fold those seen in behavioural conditions (**Figure 3.9E**). Additionally, NO production kinetics remained similar to control, slowly increasing to peak production at 48 hpi (**Figure 3.9F**). Although increased holding temperature appeared to slightly accelerate NO production, it fails to promote the 24-48hpi downregulation seen in behavioural thermoregulatory fish. These results indicated that while manually increasing body temperature in fixed increments can have some impact on inflammatory reactions, it is not sufficient to achieve the full range of immune-modulatory, and particularly the pro-resolution effects seen under conditions with active thermoregulation controlled by individual animals.

3.6 Behavioural thermoregulation vastly shortens cutaneous *Aeromonas veronii* infection while reducing host pathology and promoting rapid wound healing

While the zymosan self-resolving peritonitis model showed increased efficiency in the induction and resolution of acute inflammatory responses under behavioural fever conditions, my next step was to assess the potential improvements to host health in the

face of live infection. To this end, I took advantage of a Gram negative *A. veronii* dermatitis model in goldfish previously developed using *Aeromonas veronii* biovar sobria, which is classified as a highly virulent pathogen with a wide range of host specificity capable of infecting both fish and mammals (469). Behavioural tracking over two weeks post infection revealed high thermal preference between 23-26°C, though no clear decline in temperature preference was evident, possibly due to environmental acclimatization over the relatively long trials length, and due to time constraints no uninfected control fish were tracked (**Figure 3.10**). In fish, *A. veronii* infections are known to cause open sores or furuncles from which closely related *Aeromonas* species can shed up to 10^7 bacteria per hour in large salmonids (484). I measured *A. veronii* presence on the exterior of the furuncle as a measure of pathogen load and potential shedding. Infected T_{S16} fish displayed heavy lawn-forming bacterial loads at 1-4 dpi, *58 ± 20* and *23 ± 7* CFU at 7-10 dpi respectively, and too few bacteria to quantify at 14 dpi (**Figure 3.11A)**. T_D fish displayed an established infection with too many bacteria to quantify from 1-2 dpi, but a strikingly earlier shift to *79 ± 62* CFU at 4 dpi, and non-detectable by 7 dpi indicating that the ability to engage a behavioural fever response promotes *Aeromonas* clearance 7 days (50%) faster than T_{S16} controls **(Figure 3.11A)**. One possibility of how fever may control infection is to push the pathogen past its optimal growth range to decrease proliferation within the host. I found that this was not the case in this model given that in culture *A. veronii* replicated faster in a step wise manner as temperature increased from 16-26°C. This indicated that the immune-associated benefits of behavioural fever must greatly overshadow even an increase in bacterial replication capacity as a result of increased temperatures (**Figure 3.11B**). Due to the difficulty of isolating leukocytes from furuncles for functional analysis, I then moved to see if behavioural fever could augment cells isolated from the zymosan peritonitis model to more effectively kill *A. veronii*. Using an *ex vivo* bacterial killing assay, I saw that both T_{S16} and T_D total cell populations isolated at 16 hpi killed ~50% of bacteria after one hour of incubation (**Figure 3.11C,** solid lines). I also found that when isolated at 12 hpi, T_D cells held a 22.5%

higher mean killing capacity than T_{S16}, though this effect was not significant (**Figure 3.11C**, dotted lines). Coupled with the 2-3 fold increase in peritoneal leukocytes recruited by 12 hpi in T_D fish in the zymosan model, this data suggests that behavioural fever promotes an early influx of cells that are capable of neutralizing highly pathogenic microbes which may contribute to interrupting their early establishment within the host. While the

To analyze what may be occurring within the inflammatory site in this live infection model, cytokine expression was used to assess differences in innate responses. Similarly to our zymosan model, iNOS expression was seen to increase earlier in febrile fish peaking at ~5-fold increases at 1-2 dpi compared to 4-14 dpi in controls (**Figure 3.11D**). NO production was again associated, iNOS expression begins to decrease at 4 and 7 dpi, after bacterial load within the furuncles decline. Pro-resolution cytokine IL-10 expression in T_D fish was increased two fold or higher from 2-14 dpi, where T_{S16} fish showed little change in expression until 14 dpi (**Figure 3.11D)**. Vascular endothelial growth factor (VEG-F) expression was increased 3.5-fold on 1 dpi and holds steady at a two-fold or higher expression level. T_{S16} VEG-F remains unchanged until 4-14 dpi, where it holds a 2-fold increased expression, though with large variance. This expression of pro-resolution cytokines complement the early clearance of bacteria, as well as the wound pathology of induced furuncles in thermoregulatory fish. Similar levels of inflammation and tissue damage are seen up 1 dpi in both static and dynamic fish, with slightly less necrosis of skin tissue in T_D furuncles by day 4 (**Figure 3.11E**). Drastic differences are observed at 7 dpi, with T_{S16} furuncles continuing to erupt with active inflammation, and T_D furuncles sealed a membrane and beginning the first stages of scale regeneration. At 14 dpi, the rapid wound resolution of T_D fish continues with further scale regeneration while T_{S16} furuncles have just begun to close over with a mucous layer to begin healing. These results suggest that *in vivo*, behavioural fever or thermoregulation plays a role in not only pathogen clearance, but is also critical to inflammatory resolution and wound repair.

Table 3.1 Multivariate analysis of behaviours induced by zymosan injection in goldfish. Hourly behavioural data from all 20 zymosan and PBS fish were pooled (as seen in Figure 3.2) and analyzed for significant impacts from independent variables using a permutational multivariate analysis of variance using distance matrices.

Variable	Df	Sum of Sqares	F.Model	R^2	*p*-value	Significance level
Treatment	1	10.909	101.394	0.05726	< 0.001	***
Time	1	1.479	13.742	0.00776	< 0.001	***
Fish ID	1	23.577	219.135	0.12375	< 0.001	***
Interaction (Treatment•Time)	1	1.34	12.45	0.00703	< 0.001	***
Residuals	1424	153.211		0.80419		

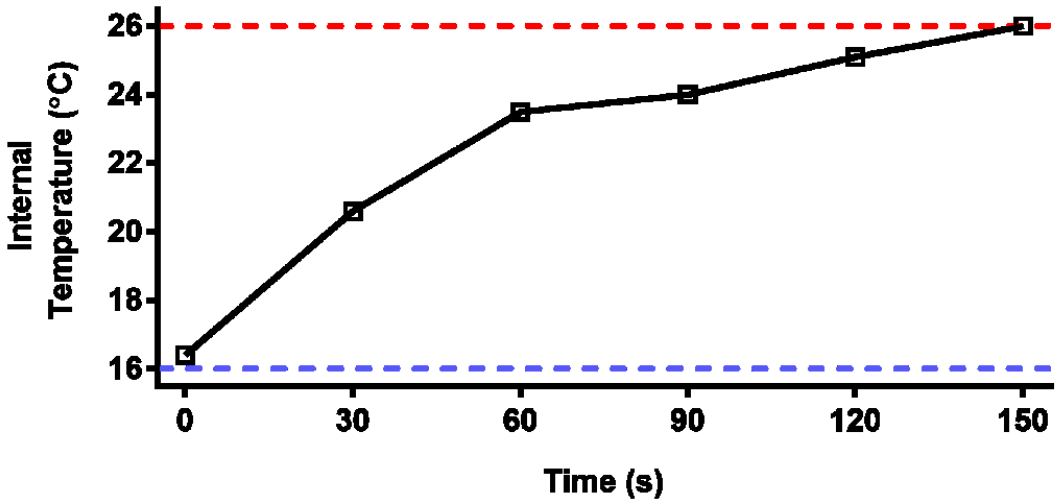


Figure 3.1 Core animal temperature changes rapidly with differential water temperature. Goldfish held at 16°C were exposed to 26°C water for 0-120 seconds before being anesthetized in 16°C (0s control) or 26°C TMS solution for 30 seconds before termination and immediate quantification of body temperature was measured by insertion of precision temperature probe into the body cavity. n=1 fish per time point, 10-13 cm in length.

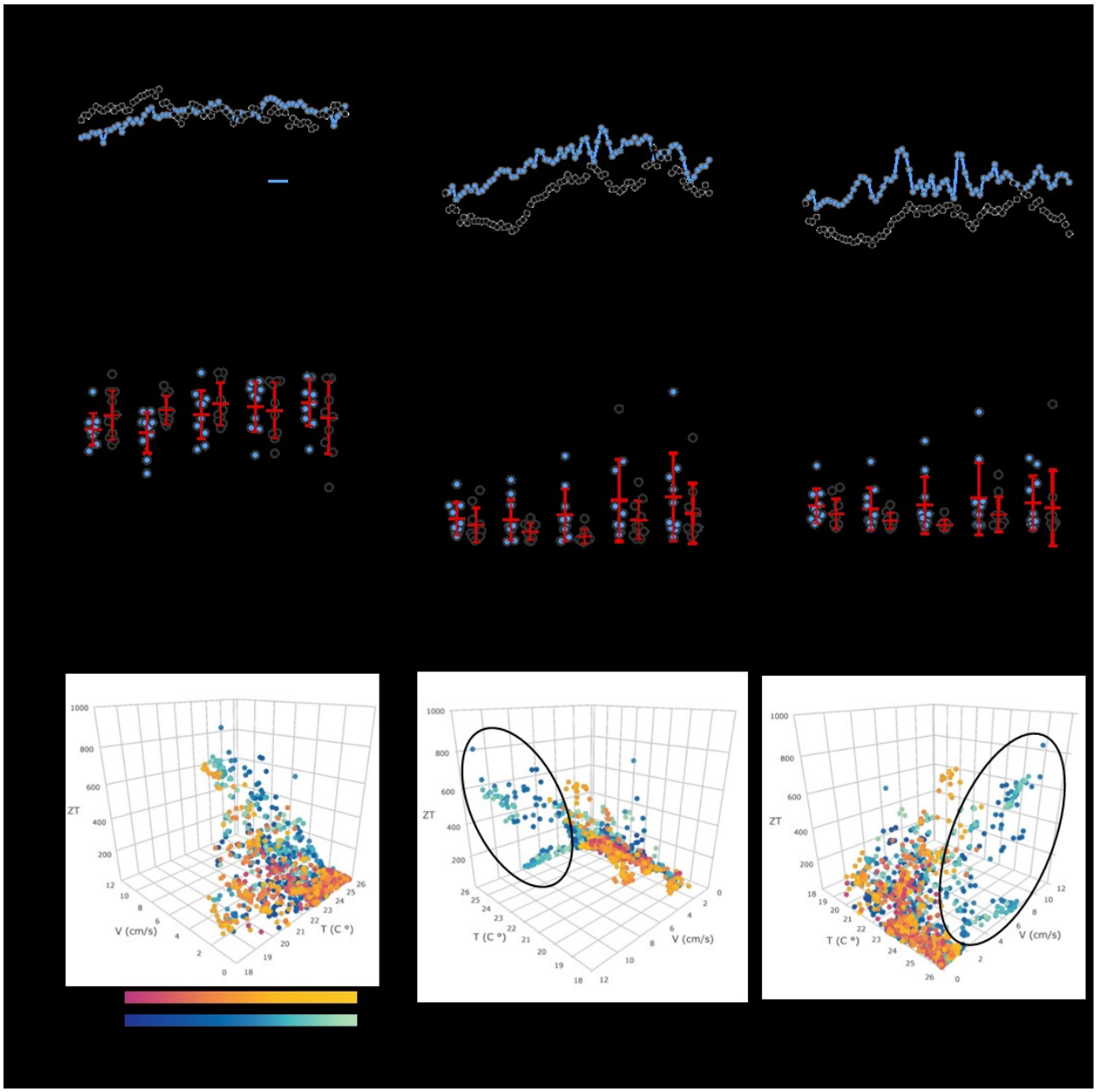

ZT
V (cm/s)
T (C °)

Figure 3.2 Intraperitoneal stimulation with zymosan generates a temporal behavioural fever in *Carassius auratus*. Goldfish intraperitoneally injected with zymosan (black) or PBS (blue) were individually placed within the annual temperature preference apparatus was recorded for 72 h using an infrared camera system. Behaviour was analyzed on a per second basis for mean temperature preference, mean velocity, and total transitions between temperature zones binned **A)** for each hour of analysis (n=10 fish for experimental and control). Mean values across replicates are indicated for each hour. Dotted lines correspond to time bins used in **B)** Mean values were calculated using per second data for each variable during respective time frames that correspond with immune kinetics seen in previous studies. Each dot corresponds to the mean value of individual replicate fish, red bars indicate group mean and standard error. ANOVA two way of variance was calculated on each variable, with *$p < 0.05$, ** $p < 0.01$ by Sidak's multiple comparisons test. **C)** 1428 complete calculated hourly data points from all experimental and control replicates were graphed for mean temperature, mean velocity and total transitions with both zymosan injected fish (purple-orange points) and PBS control (Dark to light blue points). Dark points indicate earlier hour post stimulation, while light colors indicate later time points. Black circles highlight low movement of zymosan injected fish at high temperature.

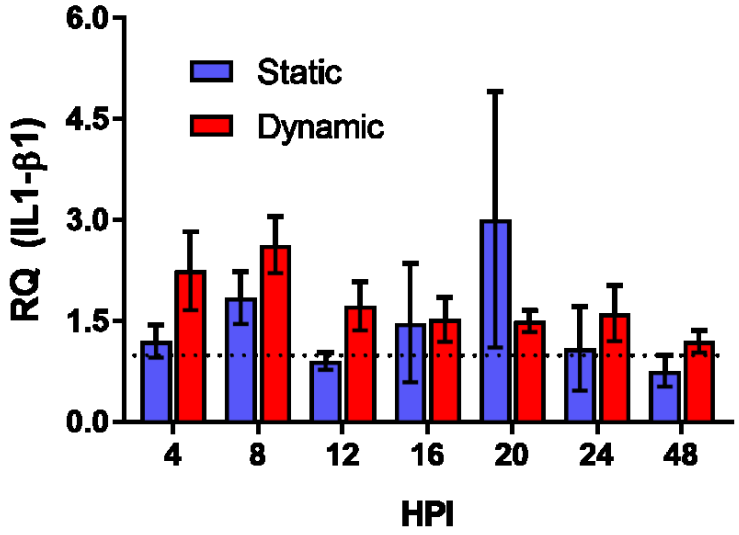


Figure 3.3 Intracranial IL1-β1 expression in goldfish. Goldfish intraperitoneally injected with zymosan were either held in static 16°C aquaria (blue) or allowed to thermoregulate within the ATPT (red). At various time points, animals were sacrificed, and whole brains were flash frozen prior to RNA extraction and generation of cDNA libraries. qPCR was used to analyze IL1-β1 content relative to un-injected controls (RQ=1 of un-injected control fish, dotted line). Black bars indicate standard error. Elongation factor 1 was used as an endogenous control. n = 5 fish per bar compared to individual control fish in 5 independent experiments. Data analyzed using a normal two-way ANOVA yielding significance between treatments ($F_{1,64} = 8.49$, $p < 0.005$) and no significant differences along time ($F_{7,64} = 1.54$, $p = 0.17$) or interaction ($F_{7,64} = 0.41$, $p = 0.90$). Sidak's multiple comparison revealed no significant pairs.

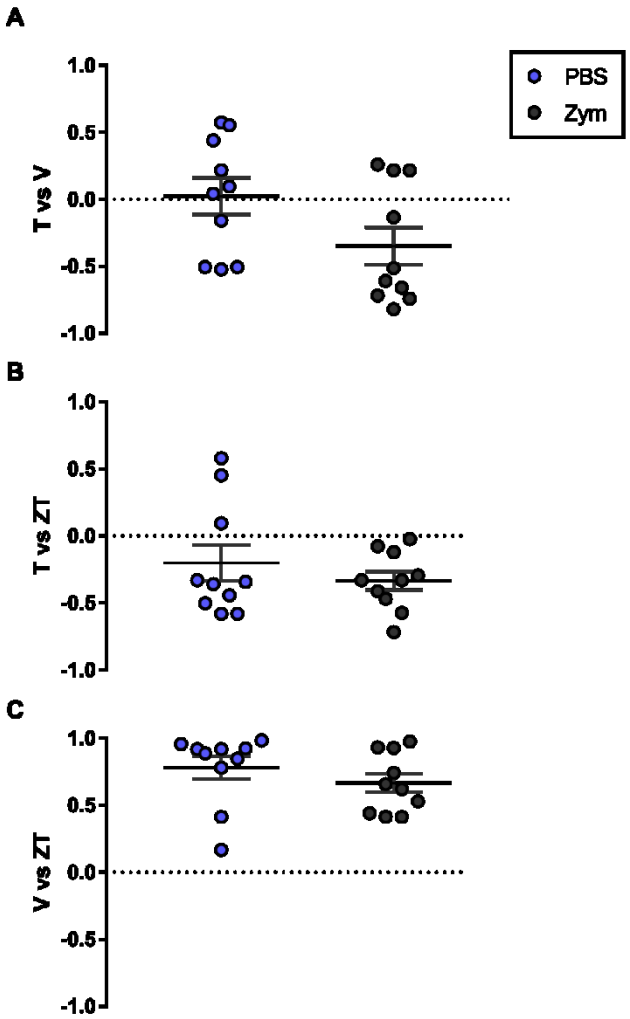


Figure 3.4 Correlation between behavioural parameters. A) 1428 Hourly binned individual behavioural data points (n = 10 each, PBS and Zym) were pooled and graphed in 3D (Figure 3.2). **B)** Coefficient of correlations were calculated for each individual fish data set between temperature and velocity (T vs V, $p = 0.06$), temperature and zone transitions (T vs ZT, $p > 0.99$) and velocity vs zone transitions (V vs ZT, $p = 0.38$) over total trials. Data was analyzed using a Mann-Whitney *U* test on ranks.

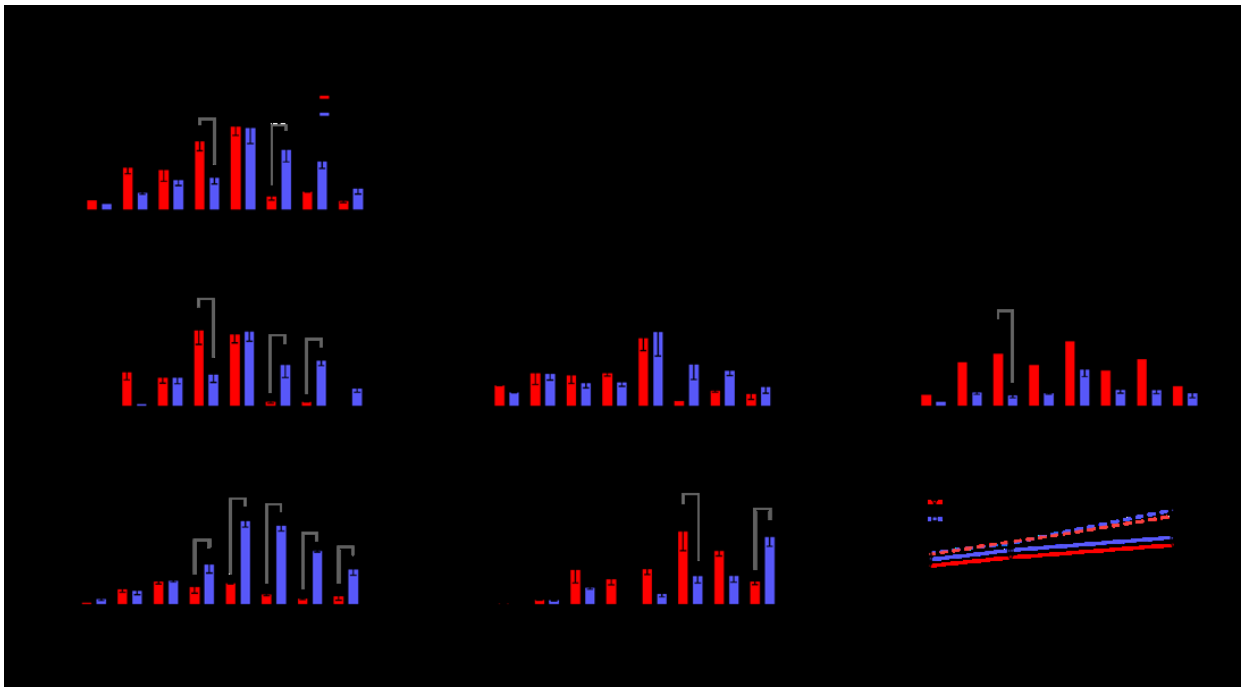


Figure 3.6 Behavioural thermoregulation promotes early recruitment of leukocytes to the inflammatory site and alters the profile of immune antimicrobial responses they express while inducing a subsequent rapid return to homeostatic levels. Goldfish acclimatized to 16°C were intraperitoneally injected with zymosan and individually placed within the annular temperature preference apparatus (dynamic, red bars) or static 16°C holding tanks (static, blue bars) before inflammatory cells were collected via peritoneal lavage and assessed for **A)** total leukocyte counts (interaction $F_{7,48} = 4.872$, $p = 0.0003$) while **B)** neutrophil (interaction $F_{7,48} = 6.66$, $p < 0.0001$), **C)** monocyte/macrophage (interaction ($F_{7,48} = 1.12$, $p = 0.28$) and **D)** lymphocyte (temperature $F_{1,48} = 25.55$, $p < 0.0001$) subpopulations were quantified using Sudan black staining and light microscopy and back-calculated using total leukocyte numbers. Flow cytometry was used to measure **E)** reactive oxygen species generation via DHR staining (temperature $F_{1,48} = 109.8$, $p < 0.0001$, interaction $F_{7,48} = 12.7$, $p < 0.0001$) and **F)** nitric oxide production via DAF-FM-DA staining (temperature $F_{1,48} = 7.67$, $p = 0.008$, interaction $F_{7,48} = 4.96$, $p < 0.0001$) respectively within total leukocyte populations. **G)** total peritoneal leukocytes were isolated at 16 hpi and co-incubated with 2:1 live GFP-*E.coli* at 16 or 26°C before aliquots were fixed at respective times and analyzed for bacterial internalization via imaging flow cytometry. **A-F)** analyzed using a regular two way ANOVA and Sidak's multiple comparisons test between pairs. Significance levels indicate * $p \leq 0.05$, ** $p \leq 0.01$ between treatment groups at respective time points. **G)** analyzed using a repeated measures two way ANOVA with Sidak's multiple comparison's test between pairs. $* p < 0.05$ static 16 vs 26°C, $‡ p = 0.05$ dynamic 16 vs 26°C. Bar tops or points indicate mean, black bars indicate standard error. (n = 4 per time point)

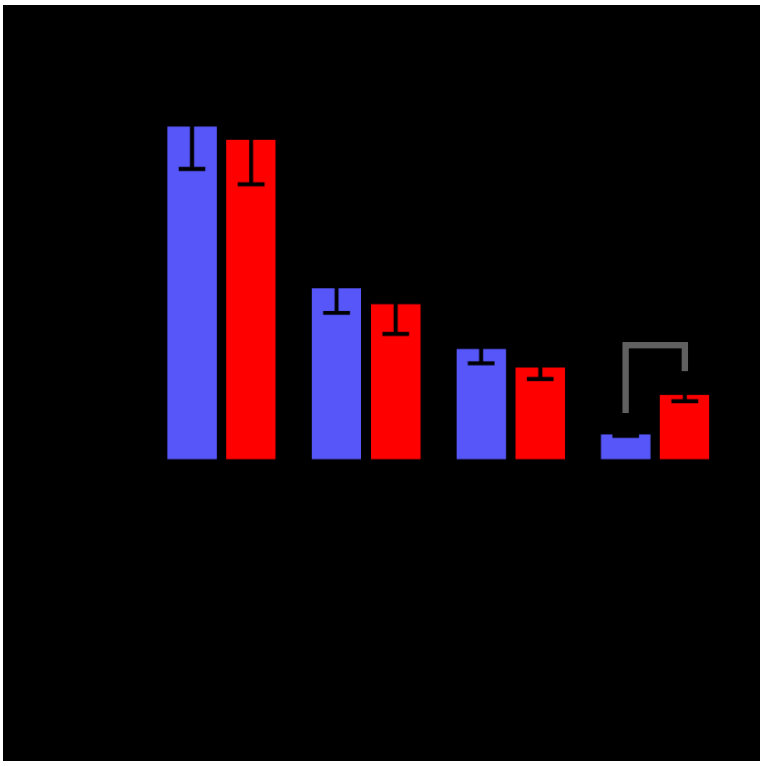


Figure 3.7 Total leukocyte infiltration over the course of inflammation. Goldfish were intraperitoneally injected with zymosan, then housed in static 16°C water (blue) or within the ATPT (red) before isolation and quantification of responding peritoneal leukocytes via peritoneal lavage. Mean values for total isolated leukocytes or leukocyte subpopulations were calculated across all lavages performed over 0, 4, 8, 12, 16, 20, 24 and 48 hpi in either static or dynamic temperature fish to represent total recruitment over the 48 h course of inflammation. Black bars indicate standard deviation. n = 32 fish per bar over 4 independent experiments. All pairs analyzed using a normal Student's t test. **** indicates a significance level of $p < 0.0001$.

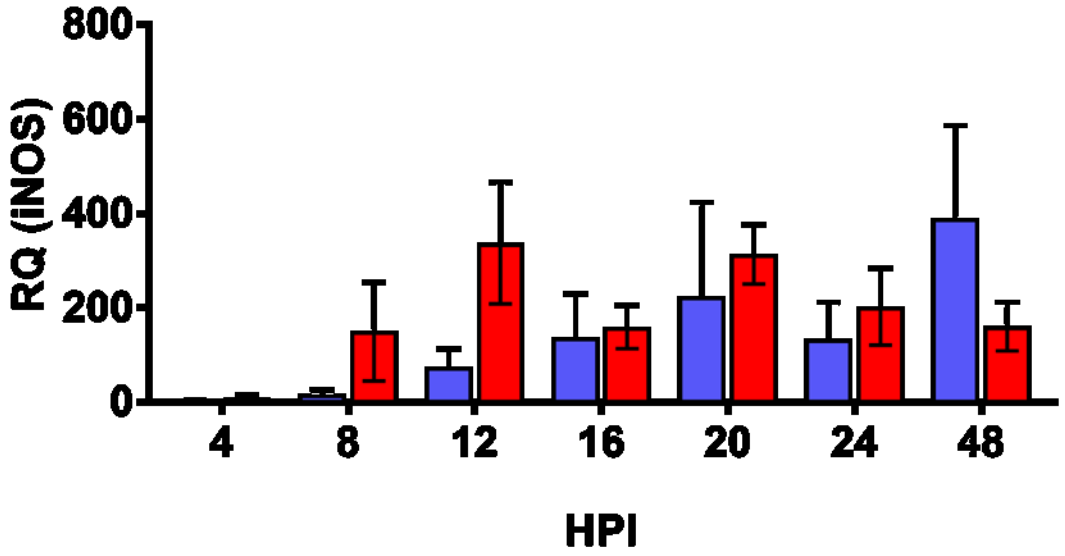


Figure 3.8 iNOS expression in peritoneal leukocytes. Peritoneal leukocytes were isolated from zymosan stimulated T_{S16} (blue) or T_D fish (red) and flash frozen prior to RNA extraction and cDNA synthesis. cDNA samples normalized by weight were analyzed for relative iNOS expression by qPCR and compared to non-injected controls (*RQ = 1*). Elongation factor-1 was used as an endogenous control. n = 4 fish per bar, compared against individual control fish in 4 independent experiments. Data analyzed using a normal two-way ANOVA ($F_{1,32}$ = 6.11, p = *0.015*). Sidak's post-hoc test revealed no significant pairs.

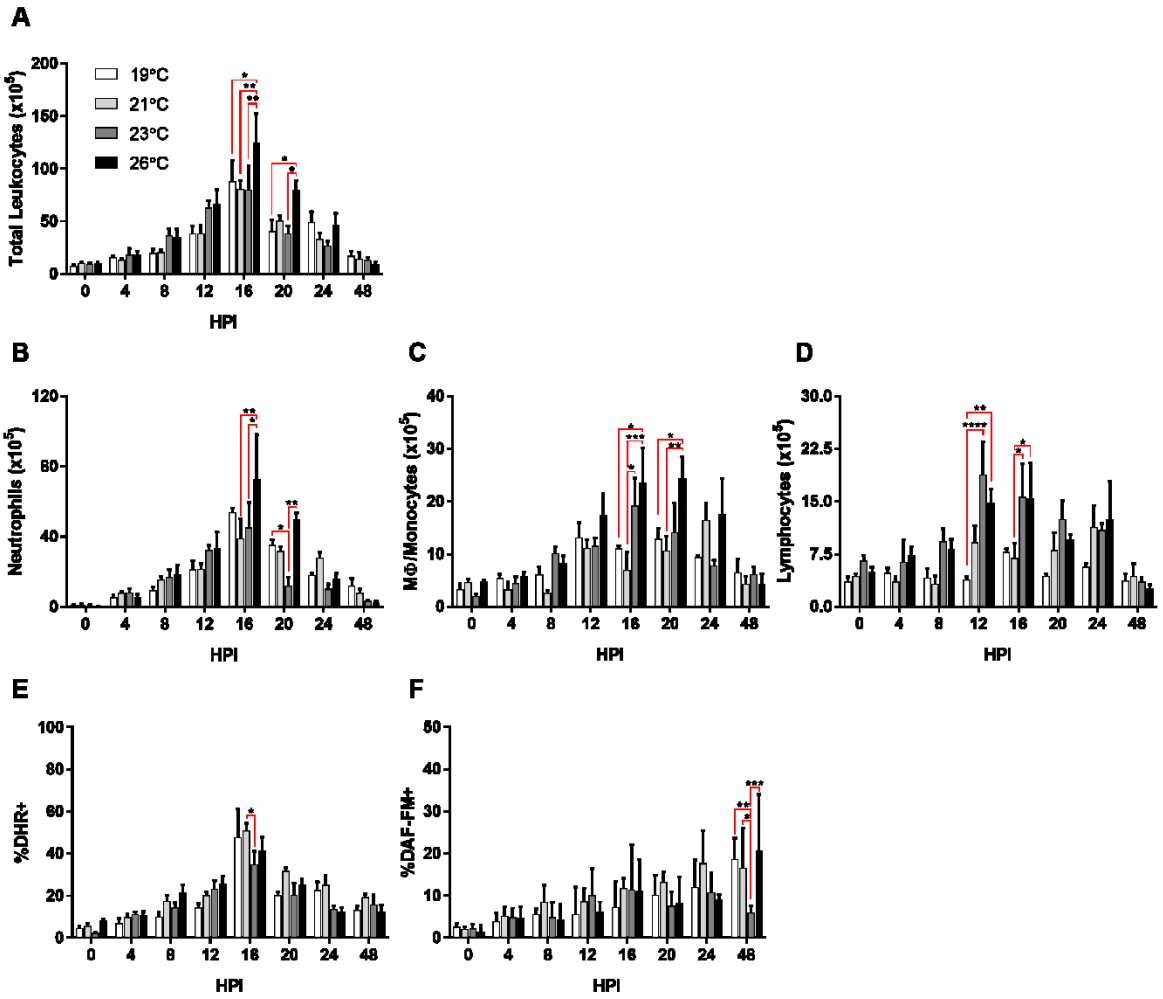


Figure 3.9 Behavioural thermoregulation and not static temperature increases are required to significantly shift recruitment kinetics or functional profiles of inflammatory leukocytes. Goldfish acclimatized to 16°C were intraperitoneally injected with zymosan and placed in either 19, 21, 23 or 26°C static holding tanks. At respective time-points, inflammatory cells were collected via peritoneal lavage and assessed for **a)** total leukocyte counts ($F_{3,96}$ = 3.81, p = 0.004) while **b)** neutrophil ($F_{3,96}$ = 2.68, p = *0*.051), **c)** monocyte/macrophage ($F_{3,96}$ = 6.39, p = 0.0005) and **d)** lymphocyte subpopulations ($F_{3,96}$ = 10.78, $p < 0.0001$) were quantified using Sudan black staining and light microscopy. Flow cytometry was used to measure **e)** reactive oxygen species generation via DHR staining ($F_{3,96}$ = 3.33, $p = 0.023$) and **f)** nitric oxide production via DAF-FM-DA staining ($F_{3,96}$ = 0.28, $p = 0.84$) respectively within total leukocyte populations. All results analyzed using a regular two way ANOVA and Sidak's multiple comparisons test between pairs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ significance levels.

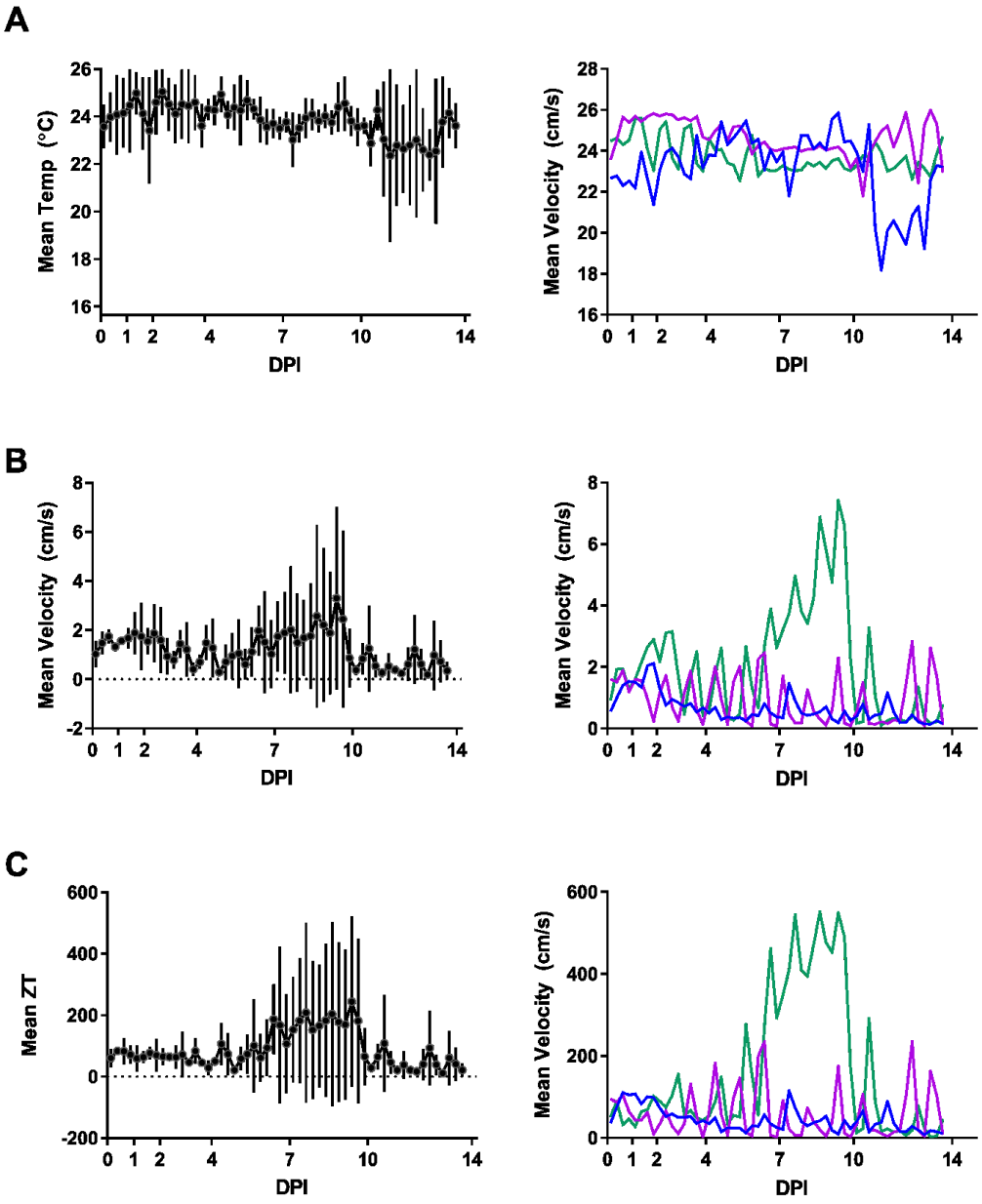


Figure 3.10 Temperature and movement behaviour of goldfish infected with live *A. veronii*. Goldfish were cutaneously infected with *Aeromonas veronii* biovar sobria and tracked within the ATPT for 14 days post infection on a per second basis. Per-second data was averaged into 6 hour time bins for **a)** mean temperature (°C), **b)** mean velocity (cm/s) and **c)** mean hourly temperature zone transitions. Both pooled (left, black dots mean, grey bars standard error) and individual trace data (right, each color corresponds to an individual fish). n = 3 fish per treatment over 6 independent experiments.

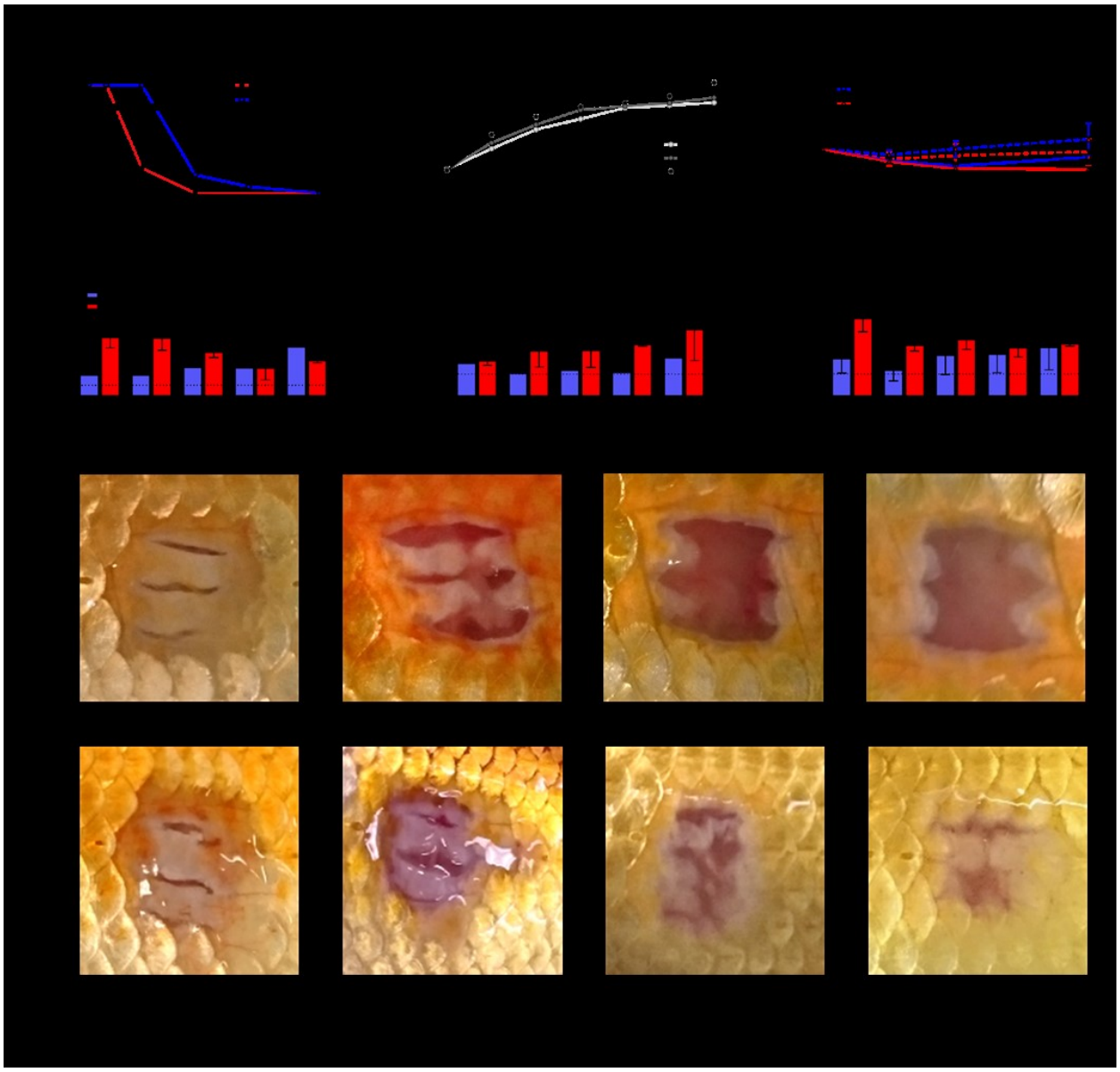


Figure 3.11 Cutaneous *Aeromonas veronii* infection induces behavioural thermoregulation which promotes increased pathogen clearance and wound resolution *in vivo* despite increased pathogen replication. Goldfish dermally infected with *A. veronii* and **A)** bacterial shedding from infected fish held in the behavioural apparatus (dynamic, *n = 3*) or static 16°C (*n = 4*) aquaria was determined by swabbing infection wound, plating onto TSA plates and assessing for CFUs on 0, 1, 2, 4, 7 and 14 dpi. **B)** *A. veronii* was grown in TSA broth at 16, 21 or 26°C, read for optical density over a course of 6 hours and CFU concentration was calculated against a standard growth curve (*n = 3*). **C)** inflammatory peritoneal cells were isolated from zymosan stimulated T_{S16} or T_D fish at 12 hpi (dotted lines) or 16 hpi (solid lines) and co-incubated with 2.5:1 *Aeromonas veronii*. Aliquots were lightly lysed and plated on TSA plates along with an *A. veronii* only control (black line) before assessment of viable bacteria through CFUs. **D)** Collected furuncle tissue was assessed for expression of inducible nitric oxide synthase (iNOS), IL-10 and VEG-F using qPCR. Dotted line corresponds to control un-infected tissue expression (RQ=1) and elongation factor 1 was used as endogenous control (*n = 3* per treatment). Expression of cells was compared to expression of cells collected from un-injected fish (black dotted line). **E)** Representative images of induced infections over the course of infection to 14dpi. (*n = 3* for each time point and treatment). All statistical results correspond to a significance level of $p < 0.05$. **B**) was analyzed with an ordinary

ANOVA two way of variance test with repeated measures and Sidak's post hoc test to look for differences between 16° vs 26° (o), 16° vs 21° (•) and °21 vs 26° (*). **C**) was analyzed with an ordinary ANOVA two way of variance test with repeated measures for differences between cells isolated at 12 vs 16 hpi for T_{S16} (*) and T_D (‡) cells respectively. (•) denotes all samples different from bacterial growth control) **D**) analyzed with ordinary two-way ANOVA using log transformed data using a Sidak's post hoc test with no significant pairs. iNOS (temperature $F_{1,20} = 5.32$, $p = 0.035$), IL-10 (temperature $F_{1,20} = 5.7$, $p = 0.026$), VEG-F (temperature $F_{1,20} = 5.82$, $p = 0.026$).

Chapter 4: Discussion and Future Directions

4.1 Overview of findings

Though fever has long been documented as a medically important response to infection, it has been classified more as a symptom to treat, rather than an innate immune response. This is likely due to the elusive nature of what effects fever might be having on the immune system and by extension, what benefits fever might be conveying to the host. Though the deeply conserved nature of infection dependant thermoregulation suggests that it somehow plays an important role in survival, relatively little is known about potential advantageous effects conferred by febrile responses. While comparative models have shown great utility in studying fever without the interference of pharmaceutical or physiological side effects of febrile blockade, most studies have focused on host survivability or gene expression due to the general lack of immunological reagents available for many endothermic model species (4,11,147). The goal of my project was to assess how behavioural fever might modulate functional immune functions and possibly uncover mechanisms by which increased body temperature confers pathogen clearance and survivability.

The main goal of my thesis project was to elucidate possible immunomodulatory mechanisms that are triggered by fever using a comparative approach. I found that while intraperitoneal injection with zymosan stimulated a behavioural fever in individual goldfish characterised by a small increase in preferred temperature, this febrile response is much more clearly defined when assessed in conjunction with measures of lethargy and temperature seeking behaviour (Figure 3.2). These analyses were possible due to the unprecedented tracking of individual animals at high temporal resolution and without the influence of schooling or territorial behaviours. These three factors combined display a distinct ‘window’ of behaviour from 8-20 hours post injection, after which fish begin to

regain increased movement and exhibit similar temperature preference to controls. These behavioural effects were seen to be significantly affected by the behavioural phenotype of each fish, treatment and time by multivariate analysis which also revealed a significant interaction between treatment and time (Table 3.1). These results supported that this model of behavioural fever in goldfish would generate a behavioural fever suitable for immunological study. Additionally, the increase of IL-1β expression within brain tissue following injection concurs with a growing body of evidence that displays a conserved febrile trigger that functions through the expression of pro-inflammatory mediators by cranial vascular endothelial cells across endotherms and ectotherms (Figure 3.3), hinting at the evolutionary homology of these responses and improving the strength of using behavioural fever in goldfish as a comparative model (66,148). This could be further supported by also measuring the production of prostaglandin E2 in brain vasculature, as this is the effector that acts on thermoregulatory neurons to alter body temperature in endotherms or thermal preference in ectotherms (66,147,485).

The ability to induce a fever by behavioural thermoregulation was strongly associated with an accelerated, early recruitment of leukocytes to the inflammatory site (Figure 3.6A). This population was largely due to an increased proportion of neutrophils (Figure 3.6 B), suggesting that behavioural fever may act to rapidly deploy first responder cells to disrupt pathogen establishment in the host, or to mount a greater response and contain an already established infection. Interestingly, fever also appears to promote the decrease of inflammatory leukocytes to basal levels (perhaps rapid efflux or apoptosis), suggesting that behavioural fever's role in modulating inflammation might be two-fold: to cause a rapid induction of innate cellular defences, but also to resolve them quickly. This rapid reduction of cells allows the total number of cells recruited over the course of inflammation to be almost identical to control fish held at static temperature (Figure 3.7), suggesting that behavioural fever generates an overall equivalent leukocyte recruitment

that is brought into the inflammatory site earlier. One exception to this was the lingering increased lymphocyte presence at the inflammatory site, which resulted in an overall two-fold higher total lymphocyte recruitment in febrile fish (Figure 3.6D, 3.7). This result is of great interest, as fish have a history of exhibiting poor adaptive responses when compared to higher vertebrates.

I found that while inflammatory leukocyte recruitment was promoted, behavioural fever acts to inhibit the high level production of ROS that has previously been seen to follow the kinetics of cellular infiltration, and together helped to define acute inflammatory processes (Figure 3.6E) (243,244). This downregulation of ROS is accompanied by an early induction of iNOS expression and corresponding production of nitric oxides indicating that behavioural fever induces a paradigm shift in the antimicrobial responses expressed in responding leukocytes (Figure 3.6F, 3.8). I also found that while behavioural fever does not prime leukocytes in a way that increased their phagocytic activity *ex vivo*, temperature itself is capable of increasing the rate of bacterial internalization (Figure 3.6G). Temperature promoted phagocytosis are likely to play a role with increased *in vivo* temperatures experienced under febrile conditions, and when coupled with earlier leukocyte numbers in the site, likely provides an early and increased overall functional phagocytic response at the site of inflammation. While these pronounced differences in innate immune responses were achieved by true behavioural fever, attempting to simulate a fever by increasing the temperature of housing water to febrile levels was not sufficient to recapitulate these effects as leukocyte infiltration kinetics and functional responses of these fish remained similar to those of control fish held at 16°C. These results suggest that the active control and fine-tuned regulation of body temperature by each individual animal is critical to be able to induce the maximal immune efficiencies that behavioural fever provides. This result is consistent with a study over four decades ago that displayed 20-80% survival rates of

goldfish injected with live *Aeromonas hydrophila* when held at increasing temperature, but 100% survival in fish that could actively control their temperatures (131).

While the zymosan peritonitis model is useful to study possible underlying immune mechanisms that behavioural fever might employ to promote increased survival as reported in many other models, it lacked the ability to assess functional outcomes on the host. To assess the effect of behavioural thermoregulation in a more natural context, I used a live infection *Aeromonas veronii* furunculation model to see if the immunological effects seen at induced by behavioural fever within the ATPT would also translate into improved outcomes under infection. Unlike the zymosan model, *A. veronii* infection failed to produce a distinct period of febrile behaviours like those seen in the zymosan model over a 14 day period (Figure 3.10). Regardless, I found that the ability to thermoregulate led to a 50% faster clearance of bacteria from the wound, despite *Aeromonas veronii* cultures growing faster at the high end of the ATPT temperature range (Figure 3.11A, 3.11B). Since obtaining functional leukocytes out of furuncle tissues for *ex vivo* analysis is extremely challenging experimentally, qPCR was used to assess iNOS expression as a marker of early nitric oxide responses and showed an early increase in iNOS expression in febrile fish (Figure 3.11D), similar to those seen in the zymosan peritonitis mode (Figure 3.8). Expression of pro-resolution factors IL-10 and VEG-F were also increased in fish that could actively thermoregulate (Figure 3.11D). This expression was coupled not only with vastly decreased wound pathology and prevention of furuncle eruption, but also a much more rapid rate of healing and scale regrowth when compared to controls (Figure 3.11E).

Overall, my data shows that behavioural thermoregulation acts as a potent regulator of teleost inflammatory responses that drives the efficient induction of immune antimicrobial responses and subsequently controls inflammation to promote superior tissue repair and an effective return to homeostasis. Though there are likely many other mechanisms by which fever acts, the ability to control body temperature appears to be critical to the efficient

clearance of a highly virulent aquatic pathogen and healing of wounds to rapidly reach a state of functional tissue homeostasis.

4.2 Thermoregulation and other sickness behaviours define the febrile response in both ectotherms and endotherms

While increased body temperature in endothermic fever has long been known to encompass many other effects such as lethargy, malaise, social withdrawal, increased sleep and decreases in appetite, behavioural fever has previously been documented almost exclusively by an increase in preferred temperature. While some studies using ectothermic fever observe very large temperature differences of up to 8°C due to segmented apparatus with significantly different temperatures, I, like others, report minor temperature preferences of only a few degrees which is similar to fevers induced in mammals (130,134,142). While this difference was unpronounced, I was able to use the unprecedented temporal resolution of my behavioural tracking to use lethargy and temperature seeking behaviour as secondary measures of 'sickness behaviours, which are becoming an increasing focus of febrile responses (482). Although behaviours like lethargy are likely to play a more minor role in conserving all energy stores for an all-out and energetically expensive febrile response, infection derived changes in appetite have recently been identified as an important mechanism in the outcome of different infection types. The Medzhitov group recently found that febrile induced fasting actually acts as an important mechanism that protects neurons from ROS mediated damage through the generation of ketones through lipolytic metabolism. Conversely, the intake of glucose was protective in viral infection by preventing neuronal damage from febrile protein misfolding or denaturation (486). This demonstrates the alteration of not only body temperature by the detection of infection, but other behaviours as well, while highlighting the importance of

these responses to host survival. Due to the conserved nature of fever, it is likely that behavioural fever might also encompass anorexic or other behaviours to alter the outcome of infection.

4.3 Fever acts as a potent regulator of innate immune responses that promotes improved microbial clearance and host tissue protection simultaneously

While fever has long been thought of as a maladaptive host response to infection or a simple response to push pathogens above their optimal growth temperature, my research using behavioural fever in goldfish joins an expanding field that suggests that fever evokes modulatory effects on various immune parameters (4,132,134). In particular, the survivability of all animals using my peritonitis and live infection models, compared to other models that result in mortality, gave me the ability to study pro-resolution effects and wound resolution effects of febrile responses (131,132,142). Some of the effects I've observed remain consistent with the hypothesis that fever acts purely as a pro-inflammatory reaction, but many also suggest that fever may also play an important role in protecting host tissues from both microbial virulence and host inflammatory reactions. While many studies supporting this hypothesis were conducted using whole body and *in vitro* hyperthermia models, or are largely based solely on molecular data, the functional leukocyte responses I observed using goldfish behavioural fever complements many of these previous findings to generate a more comprehensive view of the mechanisms that fever might employ to increase survivability (4,482).

While the febrile response has long been known to be associated with host pathology, it has also been known to promote pathogen clearance. For example, in the early 1900s an

Austrian psychiatrist named Julius Wagner-Jauregg found that tertiary neurological syphilis was cleared in patients that were co-infected with *Plasmodium*, the causative agent of malaria, after they experienced the cyclic fevers that define malarial disease (487). Though Wagner-Jauregg's work on febrile therapy garnished a Nobel Prize in 1927, the immunological mechanisms underlying this induced bacterial clearance were never elucidated, largely due to the discovery of penicillin and discontinuation of febrile therapy. While research into fever has once again emerged using comparative models, very little is known about what modulatory effects that fever may be having on immune systems to promote pathogen clearance. What is known is generally based around gene expression or other molecular approaches, with very little emphasis on functional cellular immune responses (132–134,142). To address this gap in knowledge, I used a comparative behavioural fever approach in a goldfish peritonitis model that had been previously used to study functional innate immunity.

The fever-derived shift in leukocyte infiltration kinetics that I found in goldfish likely acts to recruit cells quickly to interrupt the establishment of infections or to clear them before pathogen proliferation and thus exacerbation of the infection can occur (Figure 3.6). The early recruitment of a high proportion of neutrophils supports this hypothesis, as neutrophils are known as a first responder cells armed with potent anti-microbial effector mechanisms (Figure 3.6B). Interestingly, endothermic murine and avian models with relatively high body temperatures have been seen to have much faster rates of cell infiltration in peritonitis models, and can reach peak neutrophil recruitment by 4 and 8 hpi respectively compared to 16-18 hpi in teleost fish (244,488,489). Although much of this speed may be due to the reservoirs of neutrophils being held within peripheral blood in endotherms and in hematopoetic compartments in fish, requiring an additional step to deliver neutrophils to inflammatory sites, the shift of peak neutrophilia from 16 to 12 hpi promoted by behavioural fever suggests that body temperature may have partially evolved

as a mechanism to accelerate the deployment of antimicrobial effector cells (244). While I found that increased incubation temperature increased phagocytic capacity of inflammatory leukocytes from both febrile and control fish, it is likely that increases in temperature may also act to increase the phagocytic rate of responding leukocytes *in vivo*. This is likely due to the accelerated rates of actin and myosin filaments at higher temperatures which facilitate increased pseudopodia formation at higher temperatures (490). When paired with the rapid infiltration of leukocytes, many of which are classified as professional phagocytes, febrile hyperthermia likely causes vastly increased total phagocytic potential and therefore microbial clearance within the inflammatory site, promoting the clearance of infections before they can further establish and progress. In addition, behavioural fever did not significantly improve the functional killing capacity of inflammatory leukocytes on a cell to cell basis, further suggesting that the early influx of leukocytes caused by behavioural fever may act as a main febrile mechanism to create a more pronounced antimicrobial effect early in the course of infection.

Fever has long been regarded as a symptom of or over-response to infection stemming from rampant pro-inflammatory responses (482). However, this paradigm has shifted to febrile body temperature acting as an active regulator of immune reactions, and not only an outcome of immunity. This has been displayed by temperature dependant clearance of pathogens and upregulation of antimicrobial genes in fish, and now further supported by the temperature dependant altered immune functionality found in my data (132,133). Without the increase of body temperature, many of these immune effects are lost, indicating that increased body temperature, at least in behavioural fever, is required to achieve positive immune outcomes (482). With the evolutionary conservation of febrile responses, it is thus also likely that endothermic fever plays a similar role as an active immuno-regulator, and not only a physiological outcome of infection.

Along with a growing body of evidence based on hyperthermia, my research suggests an alternative function of fever as an inducer of pro-resolution and host-protective mechanisms (4). The first piece of evidence I found that supports this hypothesis was the rapid reduction of inflammatory leukocytes present within the peritoneum shortly after the peak of infiltration (Figure 3.6A). This rapid shut down of cellular responses likely plays a role in controlling prolonged inflammation and therefore tissue damage.

Another interesting febrile effect I found was the inhibition of high levels of ROS production in peritoneal leukocytes (Figure 3.6E). This ran counter-intuitive to the effects of increased clearance seen across many other behavioural fever models, as ROS is known to act as a potent antimicrobial effector and production kinetics have previously been seen to closely follow cell infiltration kinetics (243,244,491). Interestingly, ROS inhibition coincided with the induction of NO production nearly a day earlier than in controls (Figure 3.6D). This paradigm shift may affect the host three-fold: 1] by increasing the early expression of an alternate killing mechanism to ROS which is a common target for microbial evasion through the activity of regulatory repressors and catalases (492–494). 2] by increasing the cytotoxic capacity of present oxidative mechanisms through synergistic effects of reactive oxygen and nitrogen species. *In vitro* studies have shown that NO and H_2O_2 together had a killing capacity on bacteria several logs higher than either oxidative compound alone (388,495). When paired with a rapid influx of cells seen in febrile conditions, this synergistic effect may lead to a larger proportion of cells coming in with a much more potent and targeted antimicrobial capacity much earlier in the course of infection. 3] by reducing the production and therefore release of cytotoxic oxygen radicals. Although ROS generation is usually directed towards phagosomes, premature phagosomal maturation can lead to the extracellular release of ROS and lead to subsequent damage. Further, while nitric oxides can also damage tissues at high concentrations, low concentrations that diffuse through leukocyte membranes have been shown to convey protection from ROS mediated oxidative

damage to host tissues, adding an additional layer of protection to host tissues (388,496). One caveat to the DHR detection of ROS is that HR becomes fluorescent through electron transfer through NADPH oxidases, which can serve signalling functions separate from the generation of immune-regulated ROS. Nevertheless, DHR remains the clinical standard for the flow cytometric detection of ROS levels in patient blood samples (497). Further experiments should take advantage of newer CellROX reagents which interact directly with ROS oxide anions to become fluorescent, leading to a more accurate measure of true ROS production (498).

While these discoveries using a zymosan model of simulated infection outlined novel functional immunomodulatory effects driven by febrile responses, I wanted to assess if the same range of thermoregulation could induce the drastically improved pathogen clearance seen in previous behavioural fever studies using live infection models (131–133,142). To this end, I used an *Aeromonas veronii* infection model that simulates a natural dermal infection, as several *Aeromonas* infections in fish often present in furuncles, or erupted lesions on the skin. *Aeromonas* species are also a relevant pathogen in many teleost species, and many *Aeromonas species, particularly A. veronii* are capable of also infecting a wide range of both ectothermic and endothermic hosts (404,426). Due to this bacteria's increased growth rate with increasing temperature (Figure 3.11B) and optimal growth temperature in the range of human fever, studying this organism in the context of fever removes the hypothesis that fever is simply detrimental to pathogens by heating them beyond their optimal growth temperatures (499). Another benefit to the *A. veronii* furunculation model in goldfish is that all fish recover with zero mortality, allowing the study of resolution and healing in the presence of absence of febrile responses compared to many studies that employ high mortality models which rely on control mortality to generate survival curves.

While behavioural analysis did not reveal any clear patterns of periods of increased temperature preference or reduced movement parameters (Figure 3.10), this could be due to several reasons. First, due to time constraints in conjunction with the 14 day infection used in this model, the behaviour of mock infection control fish was never assessed for comparison. Further, the dermal nature of this infection may not induce as distinct a set of sickness behaviours when compared to the peritoneal delivery of pure antigen in the zymosan model behavioural model. It has also been shown that pathogen load is critical to inducing the severity of febrile responses (486). In addition, the low replicate number may not reveal a clear pattern, as behavioural data is inherently noisy, and the behavioural phenotype of each fish contributes to a large portion of behavioural variance (Figure 3.5, Table 3.1)(500). Regardless of these facts, the environment of the ATPT allows for a significant 6-8°C increase in mean temperature of febrile fish over static 16°C controls, but also the ability to actively fine tune internal body temperature over time.

I found that this model of induced *Aeromonas* furunculation showed similar effects to previous studies, with vastly improved rates of bacterial clearance a full 7 days (50%) earlier than controls, indicating that the range of fever I had been studying was capable of inducing extremely beneficial effects to host outcomes during infection (Figure 3.11A). Future experiments should include a serial dilution to quantify the high ends of bacterial shedding above lawn forming levels, as it is not possible with my current data to assess the maximum magnitude of bacterial shedding. Never-the-less, the correlation of this early clearance with expedited healing of the induced furuncle led to further investigation into the mechanisms that might be driving this response (Figure 3.11E). While previous attempts in extracting viable leukocytes from furuncles for functional analysis have failed, it is likely that the rapid recruitment of leukocytes to the site of infection plays a role in the rapid resolution of bacterial shedding and initiation of wound healing. While the infection was still able to establish, early pathogen engagement by responding leukocytes even a few hours earlier

could vastly change the course of infection by preventing the rapid expansion and establishment of bacteria that are capable of doubling their growth over a single hour (Figure 3.11B). Early increased iNOS expression seen during furunculation in febrile fish supports the early induction of NO responses seen earlier in my zymosan induced fever work. Thus it is likely that early NO production plays a role in bacterial clearance or host protection as outlined above.

Although the febrile range in my research actually increases *Aeromonas* growth rates instead of lowering it, there may still be febrile effects projected onto *Aeromonas veronii* that result in faster clearance and reduced pathology. A main contributor to the vastly reduced host pathology seen in febrile fish may be a lowered capacity of *A. veronii* to induce apoptosis in host cells, an excess of which can cause secondary necrosis that potentiates inflammation and expansion of bacterial lesions deeper into tissues (373,501). Further, it is likely that a shift in body temperature may also alter the expression paradigm of *A. veronii* virulence factors. Several studies have shown that pathogens with broad host specificity including *A. hydrophila*, a relative of *A. veronii*, decrease the expression of certain virulence factors at increasing temperatures not usually associated with ectothermic hosts, preventing constitutive expression outside the host would be energetically expensive and thus disadvantageous (181,434). Some studies have also found that fever causes a sequestration of available iron in both ectothermic and endothermic models (502,503). This may interrupt pathogen growth, but is unlikely to be a key effector of febrile anti-microbial effects, as many pathogens are still capable of scavenging iron from low concentration, or removing iron from host heme groups (437). Though febrile effects on pathogens may occur, my findings suggest that a much larger determinant of febrile protection is instead due to the modulation of host immunology.

Interestingly, fever also appears to promote the early expression of pro-resolution cytokine IL-10, and VEG-F, which is heavily involved in revascularization in wound healing

(See *Section 1.3.5.2.2* and *1.3.4.2.3* for more detail). While it is difficult to dissect whether this expression is actively driven by fever, or is a result of early resolution due to reduced bacterial pathology, the pronounced expression of VEG-F at 1 dpi (Figure 3.11D) suggests that fever might at least partially be responsible for the active upregulation of pro-resolution factors during inflammatory induction. While the field of fever remains relatively unexplored, there are a number of studies that have detected protective anti-inflammatory or pro-resolution effects in hyperthermic models that show parallels to febrile effects uncovered in my research. For example, it was found that heat decreased joint damage in a murine model of arthritis and that this decrease was accompanied by a heat shock protein (HSP) 70 dependant upregulation of IL-10 and a reduction in inflammatory NFκB activation (504). Heat shock has also been seen to be inhibitory to apoptotic inducing effects of TNFα, protecting host tissue cells from death induced by a pro-inflammatory mediator (505). Another interesting observation was the protection of cardiomyocytes in models of septic infection by Heat shock factor 1 dependant inhibition of pro-inflammatory genes.

The fact that many of these effects can be mediated through heat shock proteins seems like a plausible mechanism, especially given the temperature driven expression of HSPs. In addition, as HSPs are already well known to promote tissue and cell protection to temperature stress, it is not unreasonable to imagine them also evolving to convey protection from increased host defences in the context of local peripheral swelling in endotherms or whole body febrile thermoregulation. In addition, the activity of several HSPs which are present in teleost fish seem to parallel many simultaneous pro-inflammatory and pro-resolution febrile effects that I've found in my research. For example, HSP 70 has been seen to promote the early expression of iNOS and subsequent early nitric oxide responses in human macrophages, similar to the early production of NO responses that I found in febrile fish (506). In addition, knock out of HSP1 caused rapid mortality in mice infected with fever inducing doses of *Listeria* through uncontrolled expression of pro-inflammatory mediators, despite

similar pathogen loads in surviving HSP1(+/+) mice (507). This hints towards the induction of protective immunomodulatory effects mechanisms during endothermic fever and their importance to host survival. While I did not focus on HSPs, they are stress regulators present in all multicellular organisms that have been assessed and are extremely well conserved with some members from molluscs through fish and humans, sharing common functional domains and structure to the point of sharing antigenicity (508–510). The evolutionary conserved and widespread nature of this family of proteins would also support their involvement in endothermic and behavioural fevers, which share a similar level of evolutionary conservation.

Overall, my research displays the functional effects of behavioural fever acting through a two-fold host 'Sword and Shield' mechanism: to engage and clear invading pathogens rapidly while promoting the tight control and resolution of inflammatory reactions to protect host tissues. While these responses may be polarized into separate categories, both likely contribute to the rapid restoration of host tissues to their functional homeostatic state.

4.4 Static temperature cannot replicate the immunological effects induced by behavioural thermoregulation

While thermoregulation is an integral component of fever and plays an important role in the modulation of host inflammatory reactions, it appears that body temperature must be tailored to each individual as static increases in housing temperature post-injection with zymosan failed to replicate most of the major immunological effects that I found to be caused by behavioural thermoregulation in goldfish (Figure 3.9). This data came as a surprise, as several previous publications studying the effect of 'behavioural' fever first assess potential temperature increases exhibited in their model organism by their respective infections or immune challenge, then simply compare responses in animals held at various

temperatures (131,134). My data suggests that while many of these studies report significant differences in their models, that there might be more pronounced, or entirely additional febrile modulations within a host undergoing true behavioural fever. Using the advantage of high resolution tracking of individual fish, I can follow the thermal preferences of each individual fish over time, instead of observing group temperatures over time (132,133). This revealed that while mean temperature increases in febrile fish, there is still roughly 2°C range of temperature preferences exhibited by each animal. It is possible that there are nuanced mechanisms that influence thermal selection in a temporally fluid fashion to maintain the tight regulation of body temperature to alter innate immunity. Fine-tuned regulation of temperature by ectotherms might be seen as a homologous mechanism to continually breaking fevers in humans that likely act to regulate various immunomodulatory effects by controlling body temperature. In addition, it is widely known that a range of febrile temperatures can be exhibited in different patients by the same infection, suggesting that endothermic body temperature adjusts itself to tailor responses to the individual. Interestingly, a similar behaviour has been noted in birds, which undergo fever at night when it is cooler, but not during the day when it is hot to regulate body temperature during infection (511). Regardless of possible mechanisms, it appears that the individual fine-tuning of body temperature is likely to play a critical role in the induction of fever induced immunomodulation.

4.5 Febrile recruitment of lymphocytes

Though my work focused on the effect of behavioural fever on inflammatory processes driven by the innate branch of immunity, the over two-fold increased recruitment of lymphocytes in febrile fish opens a door of interest into the effect of behavioural fever on the adaptive wing of immunity (Figure 3.6D). Lymphocytes are a well-established cell population in teleost fish, and are known to generate memory responses in a homologous manner to mammals. However, the memory responses in fish are generally quite weak

when contrasted to those of higher vertebrates (512). This is hypothesized to be due to the lack of lymph nodes or other organized lymphoid tissues in fish, as well as several other mechanisms highlighted in *Section 1.3.4.5* of my literature review, like low antibody affinity and affinity maturation of antibodies. The increase in lymphocyte recruitment seen in my research may serve as a mechanism to improve the responses of teleost adaptive immunity by increasing the amount of lymphocytes exposed to antigens present within the site of infection. A similar effect has been seen in mammals where fever-range temperature increases have been shown to increase lymphocyte tracking to secondary lymphoid tissues by the upregulation of certain adhesion molecules, highlighting the possibility that fever also influences the adaptive wing of immunity, and not solely the innate (513). In fact, higher acclimatization temperatures have been associated with increased antibody concentration in the sera of brown and rainbow trout, though only total antibody concentration and not the generation of antigen specific immunoglobulins was assessed (514). Regardless, this study further supports that temperature might impact antibody responses even outside the context of behavioural fever. This becomes relevant with both the study of adaptive responses in fish and the generally poor efficacy of vaccines in teleost fish which are all generally done within regulated static water temperatures (515,516). My research suggests that incorporating behavioural fever into further studies into the memory responses of lower ectothermic vertebrates might uncover mechanisms that might not only improve our understanding of the evolution of adaptive responses in vertebrates, but also improve the efficacy of vaccines in economically important aquacultural settings.

4.6 Future directions

4.6.1 Fever induced pro-resolution and tissue protective mechanisms

In my work I showed that behavioural fever can lead to the rapid clearance of *Aeromonas* infection and a vastly improved wound healing. While I suspect that much of this improved

healing is due to rapid pathogen clearance by the accelerated recruitment of leukocytes and activation of alternate killing mechanisms, I also found early increases in the expression of pro-resolving cytokine IL-10 and healing factor VEG-F early in the course of infection (Figure 3.11D). This strongly suggests that behavioural fever is also responsible for deploying protective mechanisms to reduce tissue damage during accelerated bacterial clearance. While protective or pro-resolving effects have been seen in several studies involving heat shock, no studies have directly assessed protective mechanisms deployed by fever using behavioural models (4). It has been recently shown that teleost neutrophils can induce the production of anti-inflammatory lipid mediator lipoxin A4 in a homologous manner to mammalian counterparts (244). Given the large early influx of neutrophils that I found in the peritonitis model under febrile conditions, it seems plausible that these cells might also be playing a role in the early resolution of inflammation. The measure of apoptosis at the site may also be relevant. Given the rapid reduction in inflammatory leukocytes present in the peritoneum after 20 hpi and the fact that the uptake of apoptotic bodies drives inflammatory resolution and wound healing responses in wound macrophages, this presents another possible mechanism for the promotion of protective or wound healing effects under fever.

4.6.2 The contribution of Heat Shock Proteins to febrile effects

While my work highlights some of the main effects that behavioural fever has on functional innate immunity, little is still known about the mechanisms that potentiate febrile responses. Many of the immunomodulatory effects that I found during my work on goldfish behavioural fever have similar functional activity as those promoted by Heat Shock Proteins including the early induction of nitric oxide responses, the induction of IL-10 expression and tissue protective effects during inflammation (504,506,507,517,518). Given the action of HSPs in temperature related stress, I find it highly likely that they might also play a role in potentiating febrile modulation of host physiology, as they would likely be induced upon the

generation of fever. The involvement of HSPs in fever would also help to account for the dual anti-microbial and protective effects of behavioural fever highlighted in my work and in the literature. In addition, the incredibly conserved nature of HSP structure and function across evolution would be consistent with the range of conservation also seen with febrile responses, and thus increases the likeliness of HSPs as a group of candidate molecules involved in driving febrile mechanisms. Specifically, HSP-1 (also known as heat shock factor 1) may be a good stepping stone to look at as it acts as a regulator for heat shock responses with a regulon of 165 genes and has been identified in teleost fish (519,520). Rates of nuclear translocation of this factor could be used to identify a role of HSP-70 in immunomodulation, though this technique is limited by reagent availability in fish, however due to the evolutionary conservation of HSPs, cross reactivity of other commercially available antibodies is a possibility. HSP-70 may also be a good target to assess as a potential regulator of temperature dependant immune-regulation, as it is highly conserved across mammals, fish and molluscs. HSP-70 has been seen to increase iNOS expression and NO production in mouse macrophages which mirrors results seen in both my zymosan peritonitis and live *Aeromonas* infection models (110). Using modern CRISPR knockouts of HSP-70 or HSP-1 on fish allowed to thermoregulate could also help to delineate the possible role of HSPs in controlling innate immunity, as knockouts should show some type of altered or lowered immunomodulation when compared to wildtype. This study however, is limited by the lack of annotated goldfish genomes, and may be better approached in another more established model, such as zebrafish.

4.6.3 Febrile effects on lymphocyte recruitment and downstream antibody generation

Adaptive immune responses in teleost species have been known to be relatively weak when compared to mammalian responses. This is thought to be due in part to several factors including a lack of lymph nodes or organized secondary lymphoid tissues, little to no affinity

maturation or isotype switching (281,286,291). However, the two fold increased lymphocyte recruitment I found in febrile fish suggests that behavioural fever might also play a role in forming memory responses in lower ectothermic vertebrates (Figure 3.6D). I predict that this recruitment of leukocytes might help to improve antibody titres in fish, and may play an important role in the generation of these responses in nature. The characterisation febrile effects on the recruitment of specific lymphocyte populations and resulting antibody titres in response to vaccination is already being carried out by C. Thomson and D. Torrealba in the Barreda lab.

4.7 Relevance

4.7.1 Evolutionary biology

Fever has long been associated with disease in humans, but more recently has been identified to promote survival to infection across a vast array of endothermic and ectothermic organisms. My work on behavioural fever will help to increase our understanding of the febrile response and the underlying mechanisms that it engages to promote host survival during infection. Studying febrile responses from an evolutionary perspective as a highly conserved physiological response to infection will help to increase our knowledge of the critical roles that fever plays in the modulation of host immunity.

4.7.2 Aquaculture

Due to high density and transmissibility of pathogens through water, outbreaks and often mass die-offs are one of the greatest challenges to efficient aquaculture. Expanding our understanding of teleost immunity and the modulation of immunity through temperature may allow for the development of not only novel control and treatment methods, but possibly also improved vaccination strategies to protect fish stocks from present and emerging pathogens.

4.7.3 Medical Research

Currently, the suppression of non-extreme fever is commonly prescribed to in clinical settings despite mounting evidence that this practice often increases pathology and mortality to infection (1,100,101). Expanding our knowledge of beneficial febrile mechanisms will further define it as a critical immunomodulatory response to combat infection and protect against associated pathology instead of a symptom of disease. My work highlights the benefits of fever not only as a mechanism to accelerate inflammatory responses, but also as a regulator of inflammation that promotes improved tissue protection and healing. This could lead to a paradigm shift in how fever is perceived within the medical community and potentially decrease mortality due to infection, while opening the door for the therapeutic potential of febrile responses.

4.8 Summary

The goal of my research was to characterize the effect of fever on functional acute inflammatory processes. Using a comparative model of behavioural fever, my work has further expanded the role of thermoregulation as a beneficial response to infection, acting in dual 'Sword and Shield' mechanism by rapidly deploying innate antimicrobial responses to promote efficient pathogen clearance while also protecting host tissues from inflammatory damage and subsequent pathology. This work underlines the evolution of temperature control as a key modulator of immunity and opens the door to further research that may lead to novel infection control strategies in not only aquaculture, but also public health.

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