Analysis of the acute phase response in goldfish (*Carassius auratus* L.) infected with *Trypanosoma carassii*

by

Nikolina Kovacevic

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Department of Biological Sciences
University of Alberta

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ABSTRACT

The innate immune response is a fundamental defense mechanism in bony fishes and a crucial component of innate immunity is the acute phase response (APR). The APR is a systemic and/or local response to any injury, infection, or trauma and is characterized by a change in the blood composition of acute phase proteins (APPs). In both veterinary and human medicine, APPs are well characterized, however the functional significance of fish APP orthologs has not been fully elucidated.

My master's research focused on the immunological interactions between *T. carassii* and its goldfish host with a focus on the APR. The gene expression profile of several APPs in the kidney, liver and spleen of *T. carassii* infected goldfish was examined during the acute and chronic phases of infection. The genes encoding for C-reactive Protein (CRP) and Serum Amyloid A (SAA) were highly expressed in the tissues of goldfish during the course of *T. carassii* infection. Consequently, recombinant goldfish CRP and SAA were generated and functionally characterized.

Recombinant goldfish CRP (rgfCRP) enhanced complement-mediated killing of trypanosomes *in vitro*, and the lysis of the parasites was enhanced after addition of immune serum to cultures. rgfCRP did not affect the production of reactive oxygen and nitrogen intermediates by monocytes and macrophages, respectively. Furthermore, unlike mammalian recombinant CRP, rgfCRP did not act as an opsonin to enhance phagocytosis of *T. carassii* by macrophages.

Recombinant goldfish SAA (rgSAA) treated monocytes and macrophages exhibited differential gene expression of select immune genes. rgSAA induced gene

expression of both pro-inflammatory (TNFα1, TNFα2) and anti-inflammatory cytokines (IL-10, TGFβ) in monocytes, and IL-1β1 and SAA gene expression macrophages. rgSAA was chemotactic to macrophages and neutrophils, but not monocytes. rgSAA had no effect on respiratory burst in monocytes, however, it suppressed nitric oxide production in macrophages exposed to heat-killed *Aeromonas salmonicida.* rgSAA displayed antibacterial properties against *Escherichia coli* in a concentration dependent manner.

The results of my thesis research present the first comprehensive analysis of the acute phase response during the course of a protozoan infection of bony fish. Additionally, it also presents a first comprehensive analysis of two major acute phase proteins, CRP and SAA, in bony fish.

PREFACE

This thesis is the original work by Nikolina Kovacevic and conducted under the supervision of Miodrag Belosevic. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Research Ethics Board, Project entitled "Innate Immunity in Bony Fish", protocol # AUP00000069. The animals in this study were kept in the research facility according to guidelines set by Canadian Council of Animal Care (CCAC).

Portions of Chapter 4 and 5 and Chapter 6 of this thesis have been published or are in review. The published manuscript is: Kovacevic, N., Hagen, M.O., Xie, J., and Belosevic M. 2015. "The analysis of the acute phase response during the course of *Trypanosoma carassii* infection in the goldfish (*Carassius auratus* L.)". *Developmental and Comparative Immunology* vol. 53, issue 1, 112-122. The submitted manuscript is: Kovacevic, N., and Belosevic, M. 2015. "Molecular and functional characterization of goldfish (*Carassius auratus* L.) serum amyloid A". *Fish & Shellfish Immunology* (in review) (Chapter 6 of the thesis).

The research presented in this thesis represents the first comprehensive analysis of the acute phase response during the course of infection of bony fish with a protozoan parasite. It is also the first comprehensive analysis of the molecular and functional responses of major acute phase proteins, C-reactive protein and serum amyloid A. I was responsible for data collection, analysis and writing of the manuscripts. Hagen, M.O. assisted in parasite infections and collection of tissue samples and Xie, J. assisted with collection of immune cell populations for use in quantitative-PCR analysis. Belosevic M. was the supervisory author and involved in formation of concepts and writing of the manuscripts.

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LIST OF ABBREVIATIONS

A_λ – Absorbance at wavelength
A2M – alpha-2-macroglobulin
AAT – alpha-1-antitrypsin
Ab – antibody
ACT – alpha-1-antichymotrypsin
AGP – alpha-1-acid glycoprotein
AMP – antimicrobial peptide
ANOVA – analysis of variance
APP – acute phase protein
APR – acute phase response
A-SAA – acute phase serum amyloid A
BSA – bovine serum albumin
C1q – recognition component of classical pathway
cDNA – complementary DNA
CFU – colony forming units
CRP – C-reactive protein
Cp – Ceruloplasmin
CTL – cytotoxic T-lymphocyte
CR1 – complement receptor 1
C-SAA – constitutive serum amyloid A
Cu – copper
DAF – decay-accelerating factor
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
dpi – days post infection
DSS – di-succinimidyl suberate
EF-1α – elongation factor 1 alpha
ES – excretory/secretory
EU – endotoxin units
FACS – fluorescence activated cell sorter
FBS – fetal bovine serum
FCA – Freund's complete adjuvant
FCA – Freund's incomplete adjuvant
Fe – iron
fMLP – N-formyl-methionyl-leucyl-phenylalanine
GFS – goldfish serum
GPI – glycosyl phosphatidyl inositol
FITC – fluorescein isothiocyanate
FPRL1 – formyl peptide receptor 1
Hb – hemoglobin
HBSS – Hanks Balanced Salt Solution
HDL_3 – high-density lipoprotein
Hep – Hepcidin
HK – heat-killed
HI – heat-inactivated

Hp - Haptoglobin
HRP – horseradish peroxidase
IFN – interferon
Ig – immunoglobulin
IL – interleukin
IPTG – isopropyl-β-D-galactopyranoside
kDa – kiloDalton
LAL – Limulus amebocyte lysate
LB – Luria-Bertani
LBP – LPS binding protein
LPS - lipopolysaccharide
MAC – Membrane attack complex
MBL – mannose-binding lectin
MCP – membrane cofactor protein
MMP – matrix metalloprotease
mRNA – messenger ribonucleic acid
MWCO – molecular weight cutoff
NCC – non-specific cytotoxic cell
NCS – newborn calf serum
NBT – nitro blue tetrazolium
NK – natural killer cell
NMGFL-15 – incomplete medium used for PKMs
NO – nitric oxide
NLRP3 – NLR family pyrin containing 3
O.D. – optical density
ORF – open reading frame
PAMP – pathogen associated molecular pattern
PBLs – peripheral blood leucocytes
PBS – phosphate buffered saline
PC - phosorylcholine
PCR – polymerase chain reaction
PE - phosphoethanolamine
PEG – polyethyleneglycol
PTX3 – pentraxin 3
PKM – primary kidney macrophages/monocytes
PMA – phorbolmyristate acetate
PRR – Pattern recognition receptor
Q-PCR – quantitative PCR
RACE – rapid amplification of cDNA ends
RBC – red blood cell
RNA – ribonucleic acid
ROI – reactive oxygen intermediates
RT-PCR – reverse transcriptase polymerase chain reaction
SAA – serum amyloid A
SBTI – soybean trypsin inhibitor
SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM – standard error of mean
TBS – tris buffered saline
TDL-15- incomplete medium used to culture *Trypanosoma carassii*
TGF – Transforming growth factor
T_{h1} – Helper T-cell population (induces T-cell response)
T_{h2} – Helper T-cell population (induces B-cell response)
TLR – toll-like receptor
TLF-1 – trypanosome lytic factor-1
TLTF – trypanosome lymphocyte trigger factor
TMS – tricaine methane sulfonate
TNF – tumor necrosis factor
TS – trans-sialidase
TTBS – tris buffered saline containing Tween 20
UTR – untranslated region
VSG – variable surface glycoproteins
WAP65 – Warm-acclimation protein 65

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

1.0 Introduction

The innate immune response is a fundamental defense mechanism in all animals, playing a critical role in rapid recognition and initiation of antimicrobial defense towards infiltrating pathogens [1]. Acute phase response (APR) during early infection involves the synthesis of acute phase proteins (APPs), known to play a crucial role in immune processes and pathogen elimination in higher vertebrates [2,3]. A large repertoire of APPs exists, which work in concert to modulate immune responses and inflammation, and aid in the recovery and repair of damaged tissue [3]. In human and veterinary medicine, APPs are well characterized and are currently used as a diagnostic and prognostic tool for assessment of infection and inflammation [4,5]. However, in fish the functional significance of APP orthologs has not been fully established.

In aquaculture settings, where fish are in overcrowded and stressful conditions, infectious diseases cause devastating economic losses [6]. Research has focused on prevention and treatment of infectious diseases, however the latter has become inconsequential as government approval for therapeutics is difficult to obtain. Therefore, elucidating fish defense mechanisms has become the focus of research in fish. In contrast to bacterial and viral infections, parasitic infections are more difficult to diagnose due to lack of specific diagnostic tools [7]. Adapting parasites to laboratory models is a difficult task, further limiting the knowledge of innate immune responses during parasitic infections [8].

Our laboratory has developed a natural host-parasite model system that is be used to assess numerous parameters of this relationship. *Trypanosoma carassii* is an extracellular protozoan parasite with a wide host range and ability to infect a variety of economically important fish [9]. Infection with *T. carassii* results in significant morbidity and mortality, especially in aquaculture setting, where prevalence of infection can reach 100% [10]. Despite its high prevalence, little is known about the innate defense mechanism to *T.*

carassii in bony fish. *T. carassii* is a naturally occurring parasite in goldfish (*Carassius auratus* L.), making this fish species an excellent model to study interactions. There are several advantages for using goldfish. They include: (1) goldfish are hardy and relatively inexpensive; (2) well-characterized immune cell *in vitro* cultivation system; (3) availability of immunological reagents; and (4) *T. carassii* can be cultured *in vitro* and subsequently be used to infect fish *in vivo* [9,11,12].

My thesis research focused on the characterization of the acute phase response (APR) of goldfish infected with *T. carassii*. This knowledge of host defense can be applied to optimize innate immune response against fish pathogens. In addition, it may enable the development and design of diagnostic tools for identification of not only pathogens, but also assays for the assessment of immunotoxcity due to exposure to chemical pollutants in the environment.

1.1 Objective of the Thesis

The goal of my thesis research is to investigate the immunological interactions between *T. carassii* and goldfish, with a focus on the fish APR. The main objective of my thesis was characterize the APR of the goldfish during the course of infection with *T. carassii.* The specific aims to my research project were to (a) Identify prominent acute phase proteins (APPs) during the course of *T. carassii* infection by examining changes in the gene expression; and (b) To clone, express and functionally characterize C-reactive protein (CRP) and serum amyloid A (SAA).

1.2 Outline of thesis

This thesis is comprised of 7 chapters. Chapter 2 is a literature review summarizing innate immunity in fish with an emphasis on current knowledge of the acute phase response and acute phase proteins, and their role in trypanosome infections. Chapter 3 contains detailed materials and methods I used throughout my thesis research. Chapters 4 to 6 contain research findings of my thesis research, and Chapter 7 is a general discussion of research findings presented in the thesis.

CHAPTER 2

LITERATURE REVIEW

2.0 Introduction

The immune system in vertebrates is divided into two branches: innate and acquired/adaptive immune responses [13]. The innate immune responses plays a critical role in rapid recognition and initiation of antimicrobial defense towards a broad-spectrum of infiltrating pathogens or internal stimuli, whereas the main features of the adaptive immune system involve immunological memory, and generation of specific antibodies to "non-self", modified "self", and pathogen-derived molecules [1,14]. This literature review focuses on the innate immunity of fish with a specific focus on the acute phase response (APR) of vertebrates and APR in trypanosome infections.

2.1 Innate Immunity in Fish

The innate immune system is comprised of three main components: physical barriers, cellular factors and soluble factors.

2.1.1 Physical Barriers

Physical barriers include the epithelial and mucosal barrier of the integument of the fish (skin, gills and gastrointestinal tract).

Lying at the interface between fish and surrounding water is the skin. The skin of fish has a unique structure, differing from mammals, and is composed of living, non-keratinized epidermal cells [15]. Mechanisms such as wound repair are accelerated in fish compared to humans, and is attributed to migratory abilities of epithelial cells from the periphery over the surface wound, providing a mechanical barrier against opportunistic infections [16,17]. Migrating epithelial cells, which are analogous to resident dendritic cells are also capable of phagocytosis and antigen processing [18,19]. As a protective mechanism, epithelial hyperplasia results in thickening of the epidermis in response to skin parasites or irritants [19,20].

The gills comprise a large surface of the fish, and is one of the main portals of entry for numerous waterborne diseases such as bacterial diseases (*Aeromonas salmonicida* [21], *Yersinia ruckeri* [22,23]) or ectoparasites infections (*Ichthyophthirius multifiliis*) [24], *Neoparameoba perurans* [25]). Gills contain populations of plasma cells and leucocytes [26], and have increased expression of Major Histocompatibility Complex Class I and II in response to infections such as *N. perurans* [27,28].

Similar to the skin, the gastrointestinal tract is continuously challenged, for example with food antigens in addition to infectious agents [29]. However, the gastrointestinal tract environment has a low pH making it less favorable for some pathogens [30]. Unlike mammals, fish intestine lacks specialized structures such as Peyer's patches, however, it contains plasma cells and numerous lymphocytes such as granulocytes and macrophages [26,29,31]. If pathogens threaten the integrity of the epithelium, an inflammatory response can be elicited, however the immune mechanism of the gastrointestinal system in fish remains to be fully elucidated [29].

The integument of the fish shares a common feature, a mucosal layer, where mucus can trap microorganisms and subsequently slough to prevent colonization [1,30,32]. Mucus is composed of high-molecular weight glycoproteins, called mucins [8,15]. The composition and structure of mucus changes depending on physiological, immunological or environmental conditions [33]. For example, excess mucus production occurs in response to colonization by the ectoparasite, *Gyrodactylus derjavini* [34]. Furthermore, changes in the glycosylation of mucins occurs in response to intestinal parasitic infections, such as *Enteromyxum leei,* leading to a reduction of bacterial adhesion [35]. Lastly, mucus contains other immunological parameters such as cellular factors and soluble mediators, which will be discussed in detail in subsequent sections.

2.1.2 Cellular Factors

Different cell populations are involved in innate immunity of fish including CD8+ cytotoxic T-lymphocytes (CTL), non-specific cytotoxic cells (NCCs), and phagocytes (granulocytes, monocytes, macrophages and thrombocytes).

Cell-mediated cytotoxicity is a process where the immune system can sense and kill foreign, virus-infected or altered cells [36]. In mammals, natural killers (NK) cells are important against viral infections [37], and CTLs are important against re-infection [38]. NCCs in fish have similar properties to mammalian natural killer (NK) cells, based on their ability to lyse xenogenic cell lines [38]. However, unlike NK cells, NCCs are small and agranular [39,40]. The monoclonal antibody (5C6) used to identify NCC populations, binds a large proportion of mammalian NK cells [39–41]. Similar to mammalian NK cells, NCCs require direct cell-cell contact to cause cell lysis [40], and are capable of killing allogenic targets and protozoan parasites such as *I. multifiliis* and *Tetrahymena pyriformis* [41,42].

NK-like cell lines have been identified in channel catfish (*Ictalurus punctatus*) PBLs, and non-specifically kill target cells [41]. However, unlike NCC, the monoclonal antibody (5C6) fails to recognize NK-like cell lines suggesting they are not NCCs [41]. Furthermore, differences in cytotoxicity exists between clones and NK-like cells appear to mediate lysis through induction of apoptosis [43].

CTLs recognize specific peptides and exogenous antigens that are present on self MHC class I molecules [37]. Most studies assessing cell mediated cytotoxicity in teleosts have focused on transplantation experiments and graft rejection [44,45]. However, more recent research has identified and isolated molecules involved in CTL recognition of antigens such as MHC, T-cell receptor (TCR) and perforin [44,46,47]. In teleosts, perforin-mediated cytotoxicity by CD8+ CTLs occurs in response to intracellular pathogens or molecules expressing alloantigens [47–49]. For example, CTL mediated cytotoxicity is important against intracellular pathogens such as *Edwardsiella tarda* in crucian carp (*Carassius auratus langsdorfii*) [49].

Granulocytes include basophils, eosinophils, neutrophils, and mast cells. In mammals, basophils and eosinophils are primarily involved in defense against parasites [36]. In the past, it was believed that the equivalent of these cell lineages did not exist in all fish species [50]. However, current literature provides conflicting evidence regarding the existence of eosinophils in teleosts, which are also referred to as eosinophilic granular cells (EGC), and in some instances believed to be of the mast cell lineage [51,52].

Eosinophil-like cells have been identified in numerous fish species including goldfish [53], channel catfish [54] and zebrafish [55]. Functional studies have demonstrated that zebrafish eosinophils degranulate in response to the extract of, *Heligmosomoides polygyrus* [55]. Additionally, mast cells have been identified in zebrafish [56], and are proposed as being homologous to mammalian counterparts [57]. Unlike eosinophils, basophils are often affected by staining procedures, and therefore the occurrence of basophils in teleosts has not been convincingly established [58]. However, it has been reported that basophil-like cells were present in the head kidney of goldfish (*Carassius auratus*) [58,59]. In the future, the identification of more reliable cell markers will allow for better characterization of teleost granulocytes [57].

Similar to mammalian counterparts, fish neutrophils were characterized by their phagocytic, chemotactic, and induction of antimicrobial responses [60–62]. Unlike mammalian neutrophils, goldfish neutrophils remain viable in culture for extended periods [60], allowing for *in vitro* analysis of functional responses. For example, goldfish neutrophils are chemotactic to, and degranulate in response to *A. salmonicida* [60]. Development of transgenic zebrafish in addition to specific neutrophil markers will undoubtedly advance our knowledge of neutrophil function in fish, with respect to cell recruitment and resolution of inflammation [63].

Fish macrophages and monocytes have been identified in multiple fish species including goldfish [11,62]. Macrophages are distributed throughout the tissues, including the gills but are mostly found in the spleen and kidney. Monocytes, precursors to mature macrophages, are located primarily in the blood and kidney, and upon specific inflammatory stimuli, undergo differentiation into tissue macrophages [8]. The environmental cues determine the functional phenotype of macrophages [36]. In PKM cultures classical activated macrophages are induced by Th1 cytokines, where progenitors differentiate into monocytes and then macrophages [64,65]. Interferon-gamma (IFNγ) promotes Th1-biased responses and has been identified in teleost fish, including goldfish [65,66]. Goldfish recombinant IFNγ enhanced phagocytic capability, respiratory burst, and nitric oxide production in macrophages [65]. Alternatively activated macrophages may also be activated by the Th2 cytokine environments [64]. For

example, in common carp (*Cyprinus carpio*), protozoan parasites such as *Trypanoplasma borreli* and *Trypanosoma carassii* can modulate the polarization of carp macrophages to generate arginase and became M2-type cells [67].

Induction of potent killing mechanisms in monocytes and macrophages such as production of reactive oxygen intermediates (ROI) and nitric oxide (NO) have previously been documented [11,62]. Monocytes, however, become less potent at producing ROI as they mature into macrophages [11].

2.1.3 Soluble Factors

Soluble factors include both soluble molecules and cell-associated receptors, which can be found in the plasma or at the site of inflammation [32]. Humoral factors include cytokines such as interleukins (IL) or tumor necrosis factor (TNF), antimicrobial peptides (AMP), lyzosomes, acute phase proteins such as complement components, pentraxins or transport proteins.

Cytokines mediate signaling within the immune system, and are produced by leukocytes in addition to other cells such epithelial cells [68]. For example, pro-inflammatory cytokines such as IFNγ and TNFα2 can prime ROI and NO responses in goldfish PKM macrophages [65,69]. IFNγ is a cytokine with antiviral activity produced by Th1 cells and natural killer cells to promote Th1-biased responses [66]. TNFα is secreted by macrophages, and monocytes and is important for enhancement of antimicrobial responses [70]. Similar to TNFα, IL-1β1 mediates numerous inflammatory responses such as cell proliferation and induction of chemotactic recruitment of T and B cells, and production of IFNs [71].

Lysozyme are bacterial enzymes that break down peptidoglycan walls of bacteria, causing cell lysis [1]. They are secreted by monocytes, macrophages and neutrophils, and are abundant in lymphoid tissues and mucus [36,72]. In fish, lysozymes are an important innate defense mechanism against a variety of pathogens [73].

In fish, the innate immune response is considered a fundamental defense mechanism primarily because fish are poikilotherms, making them inefficient in

mounting acquired immune responses [74]. The consequence of this is a less diverse antibody repertoire, impaired immunological memory and slower onset of lymphocyte proliferation [32,75]. To compensate for a less-evolved adaptive immune response, a key component of the innate immune responses of fish is the acute phase response (APR), that is suggested to be more robust than that of higher vertebrates [76].

2.2 Acute Phase Response (APR)

Homeostatic mechanisms ensure an optimal internal environment in the presence of constantly changing external stimuli. Deviation from a state of homeostasis results in the induction of an orchestrated, rapid sequence of systemic and physiological changes collectively known as the APR (Fig. 2.1). The APR is non-specific and can be initiated by a variety of stimuli including, but not limited to, the following: infection, trauma, tissue injury, cancer, chronic disease and other immunological reactions [2,77–79]. A significant change in the blood plasma composition of acute phase proteins (APPs) characterizes the APR, whose main function is to eliminate the etiological cause of disruption, such as a invading pathogen, promote repair mechanisms, and ultimately return the body to homeostasis [2,80].

2.2.1 Initiation, maintenance and termination of the APR

The APR is initiated locally at an inflammatory site upon recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) [81] present on innate cells such as macrophages, dendritic cells and neutrophils [82]. Activation of these immune cells leads to the production and release of pro-inflammatory cytokines such as interleukin (IL) IL-1, IL-6, and tumor necrosis factor-alpha (TNFα), that induce the synthesis of APPs by hepatocytes in the liver [2,83]. In addition to the induction of the systemic APR response, local reactions occur that increase capillary permeability, increase infiltration of leukocytes at inflammatory sites and induces changes to the endothelium [80]. Fever, somnolence and anorexia are other phenomena associated with the APR [84]. Unlike mammals, fever in fish appears to be a behavioural

response rather than a physiological response [85], due to the arrangement of branchial circulation and ventilation within fish, and the physical properties of water [86,87].

The reported variability of APP profiles in different pathophysiological states indicates that APP induction is context and species dependent and regulated by a complex network of mechanisms [3,88,89]. The synthesis, secretion and clearance of APPs vary making clinical interpretations among different species and challenges difficult [4,76,78].

In mammals, APPs reach a peak concentration approximately 48 hours after initiation of the APR. This response declines as the organism recovers, in the absence of additional stimuli, allowing feedback mechanisms to limit the APR, leading to a resolution within 4-7 days [4]. For example, rapid removal of circulating cytokines assists in the down-regulation of the APR by reducing stability of cytokine mRNA and reducing transcription, and the release of IL-10 suppresses IL-6 [80,90]. However, if stimuli persist, such as long lasting protozoan infections, the APP production and APR response may become chronic [80,88,91,92]. In chronic infections, change in APP composition and sustained production have been reported [4]. Unlike the short term benefits of the APR, sustained chronic APR may have a negative impact on host health, resulting in side effects such as anemia, cachexia and increased risk for development of cancer [93].

2.2.2 Classification of Acute Phase Proteins

APPs are classified according to a variety of criteria including amplification of synthesis (positive APPs increase whereas negative APPs decrease), intensity of production rate (major, intermediate and minor) and function (i.e. complement components, protease inhibitors or transport proteins) [76]. Additionally, APPs can be classified according which cytokine subsets induce their expression. Type I APPs require the synergistic induction by IL-1-like cytokines (IL-1α, IL-1β, TNFα and TNFβ) and IL-6 whereas Type II APPs are induced by IL-6 [80]. In this review, APPs will be classified according to their function; however other classifications are presented in a summary of acute phase proteins (Table 2.1).

2.2.3 Major acute phase proteins

2.2.3.1 Pentraxins

Pentraxins are a superfamily of pentameric-structured proteins functioning as soluble pattern recognition molecules [94]. C-reactive protein (CRP) and Serum amyloid P (SAP) constitute the short pentraxins, which are protypical acute phase proteins. Long pentraxins including pentraxin 3 (PTX3), also exist and have pattern recognition properties when induced by inflammatory cytokines [94].

These pentraxins have been conserved throughout vertebrate and invertebrate evolution, both in primary structure and binding capabilities [95,96]. CRP was the first pentraxin identified in 1930, by its calcium dependent binding property to C-polysaccharide in humans infected with *Streptococcus pneumonia*. In healthy humans, CRP is barely detectable (<1 μg/mL), whereas during the APR serum concentrations can increase up to 1000-fold [97,98]. This increase in serum concentrations of CRP is not as pronounced in other mammalian species [99]. In fish, pentraxins only show a slight increase in concentrations during the APR [100]. In addition to differences in APR concentrations between species, differences in baseline levels of CRP exist and have been linked to polymorphisms in the CRP gene [101,102].

CRP and SAP can bind numerous ligands. For example, CRP can bind phosphorlycholine (PC), which is a moiety present on many bacteria, fungi, whereas, SAP can bind phosphoethanolamine (PE), agarose, glycans and DNA [103–106]. The binding capabilities allow CRP to recognize pathogens and act as an opsonin, causing both enhanced complement–mediated lysis and phagocytosis of pathogens. In addition, CRP binds C1q complement component and activates the classical complement pathway [107]. *In vitro,* CRP have been shown to interact with the Fc receptor of human monocytes and macrophages, inducing the production of pro-inflammatory cytokines [107–109]. However, the effects of CRP appear pleiotropic, since both pro- and anti-inflammatory effects have been described [110–112]. For example, CRP increased production of anti-inflammatory cytokines (IL-10), while inhibiting production of pro-inflammatory cytokines such as TNFα and IFNγ in an experimental mouse model [113].

Both CRP and SAP are present in human sera, sharing 51.6% amino acid identity [114]. Basal levels of SAP are higher in humans than CRP, however SAP is not involved in the APR in humans, unlike mice where it is the main APP [102,115] Some fish species possess one of either CRP or SAP, whereas other fish species possess both [30]. For example, CRP is found in common carp [99], rohu (*Labeo rohita*) [116] and Asian seabass (*Lates calcarifer*) [117], SAP is found in Atlantic salmon (*Salmo salar*) [118] and rainbow trout (*Oncorhynchus mykiss)* [119], whereas both CRP and SAP can be detected in shark (*Pangasianodon hypophthalmus*) [120], and cod (*Gadus morhua*) [121].

Although CRP has been identified in the above-mentioned fish species, the functions of CRP are not fully elucidated. However, research has demonstrated CRP of flatfish (*Cynoglossus semilaevis)* can interact with gram negative and positive bacterial pathogens, and can stimulate antibacterial functions of PBLs [114]. A pentraxin-like protein from snapper (*Pagrus auratus*) was able to opsonize beads to enhance phagocytosis [122]. In virus challenged common carp, CRP serum levels were correlated to complement activity [123].

2.2.3.2 Serum Amyloid A

Serum Amyloid A (SAA) is a major APP, whose serum levels increase a 1000-fold in humans during the APR [124,125]. SAA is a ~12-14 kDa protein that has multiple physiological roles and have been implicated in pathogenesis of several disease states such as atherosclerosis, amyloidosis, cancer, Alzheimer's disease and rheumatoid arthritis [124,126]. However, the current literature on the biological role of SAA is controversial. For example, SAA has been shown to induce production of anti-inflammatory cytokines such as IL-10 [127–129], but also pro-inflammatory cytokines such as TNFα [128,129].

SAA is an amphipathic α-helical apolipoprotein of high-density lipoprotein$_3$ (HDL_3), which has been present in organisms for over 500 million years [124]. The presence of SAA has been documented in echinoderms, and based on the high amino acid conservation of this protein throughout evolution, it is proposed as having survival-value [124,130]. It was first identified in patients with amyloidosis, where it contributed to amyloid deposits [131,132].

The SAA family includes acute phase and constitutive proteins. Acute phase SAA (A-SAA) is induced by inflammatory cues and constitutive SAA (C-SAA) is constitutively expressed [133]. C-SAA is found in humans and mouse only, whereas A-SAA has been documented in all vertebrates [125]. In mammals, multiple isoforms of SAA gene exist such that *SAA1* and *SAA2* genes encode for the A-SAA, whereas *SAA4* encodes for C-SAA and *SAA3* is a pseudogene [134]. In mice, *SAA1, SAA2* and *SAA3* encode for A-SAA, *SAA4* encodes for C-SAA and one pseudogene exists. In birds, only one SAA gene exists corresponding to A-SAA [135]. It has been proposed that invertebrates and lower vertebrates such as teleosts, possess only one SAA gene suggesting this multi-gene family may have only evolved in mammals [136,137].

Human SAA appears to behave as cytokine-like immunomodulator [138]. Addition of SAA to human monocytes can induce production of cytokines, [128,129], chemokines [139] and matrix metalloproteinases (MMPs) [140]. In addition, it's been shown to act as a chemoattractant for numerous cells such as phagocytes, T lymphocytes, ands neutrophils [141–143]. SAA has been reported to have a diverse range of receptors including TLR2 [127] and TLR4 [144] formyl peptide receptor-like 1 (FPRL1) [145] and CD36 [146], emphasizing its role in inflammation. However, SAA isoforms vary in their receptor preferences, and consequently modulation of downstream responses [147]. Human recombinant SAA has also been implicated in the activation of the NLRP3 inflammasome and found to induce the release of mature IL-1β1 in human macrophages [148].

SAA was first identified in salmonids [149]. Thereafter, it's been identified in numerous fish species such as carp [150], rainbow trout [136], and zebrafish [151]. In response to chemical mimics or pathogens, genes encoding for SAA are up-regulated in various fish species [152–156]. However, the functional role of SAA in teleosts remains to be fully elucidated.

2.2.4 Proteins involved in iron storage and metabolism

Several plasma proteins involved in the APR have the capacity to carry a variety of molecules such as hormones and metal ions including iron [3,4]. Iron is essential for

the survival and biological functions of organisms [157,158]. For example, iron-containing compounds such as hemoglobin/myoglobin and cytochromes require iron for oxygen transport or cellular energy synthesis, respectively [159]. Metabolism of iron is tightly regulated to avoid cellular damage while maintaining adequate iron stores for normal cellular processes. For example, excess iron catalyzes free radical generation which can damage proteins and lipids [159,160]. Furthermore, regulation of iron is an innate defense mechanism against pathogen proliferation. For example, infection with intracellular *Salmonella typhirium,* results in increased iron export from murine macrophages, and reduced iron availability for the pathogen [161].

2.2.4.1 Transferrin

Transferrin (Tf) is an essential glycoprotein in hosts, due to its capacity to carry the ferric (Fe^{3+}) form of iron. This Fe^{3+}-Tf complex is inert and is taken up in target organs and cells after binding to Tf receptor (CD71) (Fig. 2.2) [158]. Iron sequestration mediated by transferrin can restrict growth of pathogens, however pathogens have developed mechanism to circumvent iron-withholding strategies of the host [162]. In humans, Tf is a "negative" APP, whereas in rodents it's "positive" [163]. Conversely, in fish species it's been documented to act be both a "positive" and "negative" APP [2,164]. Interestingly, transferrin has a variety other immunological functions. For example, cleaved transferrin products in fish can activate antimicrobial responses in effectors cells [165–167].

2.2.4.2 Ceruloplasmin

Ceruloplasmin (Cp) is important in metabolism of copper (Cu) and iron (Fe). It is associated with 95% of the plasma copper, however it is also essential in iron homeostasis as shown in studies using Cp deficient mice [168]. Cp is serum ferroxidase, necessary for the oxidation of ferrous form (Fe^{2+}) into the useable ferric form (Fe^{3+}) [169]. Lack of Cp results in iron accumulation and subsequent development of a neurodegenerative phenotype [170]. Cp facilitates the transport of iron from storage to sites of synthesis, thereby contributing to regulation of erythropoiesis [171]. Furthermore, Cp scavenges oxygen radicals, preventing damage mediated by reactive oxygen

intermediates [172]. Cp is up-regulated during inflammatory states [173], infection and tissue damage in humans [173,174] but has also been observed in fish species [169,175]. Recently, the role of Cp in iron metabolism has been linked to host defense [176–178].

2.2.4.3 Haptoglobin

Haptoglobin (Hp) is a plasma protein that binds haemoglobin (Hb) with high affinity, allowing for rapid recycling in the liver [179]. By binding Hb, Hp can act as an antioxidant by preventing formation of oxygen radicals that induce oxidative damage. The binding of Hp to Hb-iron complexes, has been shown to limit iron availability to bacteria [180], preventing their normal multiplication in the host. In mammals, Hp is a "positive" APP and serves as a marker of erythrocyte destruction and ensuing anemia [181,182]. For example, Hp has been shown to be up-regulated in individuals with infections and/or during inflammation, and appears to be down-regulated in individuals exhibiting hemolysis [183]. For example, in malarial infections, Hp is up-regulated and important in removing toxic free Hb [184]. In humans, differences in Hp phenotype contribute to host susceptibility [185]. In addition to its role in iron metabolism, increased Hp expression during the APR can lead to immune cell modulation such differentiation and maturation of lymphocytes [186]. Hp has been identified in some fishes [187–189], and shown to bind Hb [189], however, its influence during the immune responses of fish remains to be elucidated.

2.2.4.4 Hemopexin

Hemopexin (Hx) binds excess heme for transport to the liver where it is recycled, and because of this function has antioxidant properties [190]. It is a "positive" APP, which restricts hemes availability and recycles iron. However Hx-Hb complexes have also been shown to serve as sources of iron for pathogens such as bacteria and parasites [191]. In teleost, the homolog of Hx is Warm-acclimation protein 65 (WAP65). Interestingly, teleosts possess two WAP65 proteins, which differ in expression and function. WAP65-1 is ubiquitously expressed whereas WAP65-2 is expressed in the liver in response to temperature change or infection [192].

2.2.4.5 Ferritin

Iron can be sequestered and stored by ferritin during immune cell activation [193]. Ferritin is regulated at the transcription and translational levels by pro-inflammatory cytokines, and has been indirectly linked to innate immune responses [194]. In mammals, ferritin molecules are comprised of a heavy (H) and light (L) chain subunits, where the H chain is important in Fe^{2+} oxidation and L chain responsible for long term iron storage [164,195]. It is up-regulated in response to inflammatory conditions and infections [196]. Interestingly, teleosts contain a heavy (H) chain and (M) middle chain, where the H chain is highly conserved throughout evolution [197], and the M chain possesses properties of both H and L chain [198]. Similar to mammals, ferritin has been shown to be up-regulated in response to infections in fish [164,197].

2.2.4.6 Hepicidin

Hepcidin (Hep) is an antimicrobial peptide (AMP) and key regulator of iron homeostasis. During infections, inflammation or excess iron concentrations, increased hepcidin is produced as a defense mechanism to limit release of iron into the body [199]. It has both antibacterial and antifungal properties. Hepcidin is a "positive" APP in fish and up-regulation has been documented in fish challenged with various bacteria [200–202], or protozoan ectoparasite [203].

2.2.5 Protease Inhibitors

2.2.5.1 Serpins

Serine protease inhibitors (serpins) are involved in controlling enzymes that trigger inflammatory processes. The most well studied serpins include α-1-antitrypsin (AAT) and α-1-antichymotrypsin (ACT). AAT has been shown to inhibit proteases such as neutrophil elastase and proteinase 3, which is released by degranulating neutrophils, thus preventing tissue damage [204]. In AAT deficient individuals, development of lung and liver disease of has been documented [205]. In addition, AAT has been shown to have other anti-inflammatory properties, such as inhibition of TNFα and superoxide production by neutrophils [206]. Furthermore, AAT has been documented to inhibit the multiplication and development of protozoan parasite *Cryptosporidium parvum in vitro*

[207]. In fish, AAT has been documented to act both a “positive” or “negative” APP. For example, anti-trypsin activity decreased in cod challenged the immunostimulant, turpentine oil[208], and the gene expression of AAT-like protein was down-regulated in rainbow trout in response to a confinement stressor [209]. Conversely, in ayu (*Plecoglossus altivelis)* challenged with the bacterium *Listonella anguillarum,* an up-regulation in genes encoding ATT was observed [210]. In mammals, ACT regulates capthespin G released by neutrophils and chymases released from mast cells [211]. ACT has not been identified in teleost species.

2.2.5.2 α-2-macroglobulin

α-2-macroglobulin (A2M) is another major and broad-spectrum protease inhibitor whose main functions include specific inhibition of proteases of prokaryotes and eukaryotes [212,213]. It belongs to the complement component superfamily of proteins [214], and has a similar primary structure to complement components C3 and C4 [215], which has also been elucidated in fish [216]. A2M has been documented in various fish species such as rainbow trout [217], common carp [218] and grass carp (*Ctenopharyngodon idella*) [219] and is a “positive” acute phase protein in response to infection with parasites [220]. Furthermore, A2M has been shown to inhibit bacterial proteases in fish [221] in addition to neutralizing parasite secreted proteases [222].

2.2.6 Coagulation Factors

2.2.6.1 Fibrinogen

Fibrinogen is significantly increased in inflammatory conditions. Its primary functions are to bind and activate a variety of immune cells, assist in formation of blood clots, and regulate cell traffic during wound repair [223,224]. Research on fibrinogen in fish is limited, however fibrinogen-like molecules have been identified and unlike mammalian counterparts, the expression of genes encoding this molecule is down-regulated in fish exposed to different stimuli [209,225].

2.2.6.2 Plasminogen

Plasminogen is the precursor to the enzyme plasmin, which is a serine proteinase important in blood clot lysis and is thus important in the resolution of inflammation and

wound repair [226]. Interestingly, in salmonids, pathogen virulence factors can activate plasminogen to promote invasiveness and adherence [23]. Plasminogen expression increases in response to bacterial and ectoparasite challenges in fish [6,227].

2.2.7 Complement Proteins

The complement system is comprised of plasma and membrane bound proteins, which initiate three convergent pathways of complement: classical, alternative and lectin pathway, leading to a common terminal membrane attack complex (MAC) [228]. The classical pathway is mediated by antibody-antigen complexes, which bind C1q, the first component of complement. In addition to antibody-antigen complexes, C-reactive protein, pentraxins, and other molecules can interact with C1q to initiate this pathway. The alternative pathway is initiated through the spontaneous hydrolysis of C3 and C3b binding to microbial surfaces [229]. The lectin pathway is mediated by mannose-binding lectins (MBLs) or ficolins, which recognizes carbohydrates associated with microbes [229,230]. Activation of all three pathways leads to C3 convertase (C3bBb for alternative, C4b2a for classical and lectin) formation, which is necessary to cleave C3 into C3a and C3b. Subsequently, C3b binds C3 convertase leading to the generation of C5 convertase (C3bBb3b for alternative, C4b2a3b for classical and lectin). C5 convertase is necessary for the cleavage of C5 into biological active fragments: C5a and C5b. C5b recruits C6 to C9 which form the MAC [231,232]. C3a and C5a are anaphlyatoxins, which support inflammation through recruitments and activation of immune cells at an inflammatory site [233,234]. To ensure a homeostatic balance between destruction of pathogens and potential self damage, the complement system is tightly regulated at the level of convertases, by numerous plasma proteins and receptors such as Factor H, Factor I, membrane cofactor protein (MCP), decay-accelerating factor (DAF) and complement receptor 1 (CR1) [233].

Fish share the same set of complement genes as mammals, birds and amphibians with the exception of some complement regulators such as Factor D, MBL-associated serine protease (MASP)-1 and MASP-3 [215]. Both the alternative and classical pathways of complement have been extensively characterized in fish [235,236]. Although fish possess functional lectin pathways [237,238], the molecular composition has yet to

be fully elucidated [237], however numerous components have been identified and characterized such MASPs [239]. C3 is the central component of the complement system and multiple isoforms have been identified in most fish species [238,240–242]. Similar to mammals, fish C5a and C3a possess chemotactic properties and the ability to induce antimicrobial functions in effector cells [243,244].

2.2.8 Other acute phase proteins

2.2.8.1 α-1-acid glycoprotein

α-1-acid glycoprotein (AGP) is a highly glycosylated licopalin, which has been documented to be multifunctional [245]. AGP can act as a plasma transport protein and binds inflammatory mediators, bacterial components and drugs [246]. In addition, AGP can modulate immune responses such as inhibiting chemotaxis, and degranulation and up-regulating production of anti-inflammatory cytokines. Furthermore, AGP has been documented to have direct antibacterial activity [245,247]. It is a major APP in humans and various mammal species [245]. AGP has been identified in viviparous fish such as surfperch (*Neoditrema ransonnetii*) [248], and homologues have been identified in Japanese flounder (*Paralichthys olivaceus*) [249], however its immunomodulatory role in teleosts remains to be elucidated.

2.2.8.2 Lipopolysaccharide Binding Protein

Lipopolysaccharide (LPS) binding protein (LBP) is an essential molecule against bacterial invasion, as demonstrated in LBP knockout mice [250]. LBP mediates transport of LPS aggregates to the surface of monocytes and macrophages [251]. Low levels of LBP have pro-inflammatory effects whereas high levels of LBP have anti-inflammatory properties [252]. LBP molecules have been identified in numerous fish species such as rock bream (*Oplegnathus fasciatus*) [253], rainbow trout [254], and Atlantic cod [255]. Similar to mammalian counterparts, LBP is up-regulated during the APR and have been shown to mediate innate immune responses such as resistance to *Aeromonas hydrophila* infection in tipalia (*Oreochromis niloticus*) [256].

2.3 Trypanosomes

2.3.1 Mammalian trypanosomes

Trypanosomes are flagellate protozoan parasites that can reside inside cells (*Trypanosoma cruzi*) or are extracellular (African trypanosomes, *Trypanosoma carassii*). They are characterized by the presence of a distinctive mitochondrion, called a kinetoplast, and therefore are grouped in the order Kinetoplastida (Phylum Euglenoza). Trypanosomes infect a variety of hosts including humans, mammals and fish species resulting in significant impacts on human welfare, agriculture, and aquaculture [257].

Trypanosoma brucei and *Trypanosoma cruzi* are two human trypanosomes that cause significant morbidity and mortality. For example, *T. brucei* that causes sleeping sickness, is highly prevalent in 36 Sub-Saharan countries and despite collaborative public and private efforts to eradicate it, 65 million people remain at risk and 200,000 are infected each year [258]. Similarly, *T. cruzi* the causative agent of Chagas disease is present in 21 Latin American countries, infecting about 6 million people and it has been estimated that approximately 13% of the Latin American population is at risk of acquiring the infection [259,260]. However, infections and risk of acquiring *T. cruzi* infection is not limited to Latin America. For example, blood transfusions or organ transplants have been documented to contribute to development of *T. cruzi* infections in Canada and the United States [261–263].

T. cruzi is an intracellular parasite transmitted to humans by via triatome bug vectors. *T. cruzi* is coated with glycosylphosphatidylinositol (GPI) glycoproteins that allow for adhesion to cells including macrophages, fibroblasts and muscle tissue [264,265]. *T. cruzi* avoids antimicrobial effector functions of immune cells such as macrophages by escaping the phagolysosome [266]. During the acute infection with *T. cruzi,* individuals are often asymptomatic which has been attributed to the inhibition and modulation of host immune responses by the parasite, allowing it to establish in the host [267,268]. Pathogenesis associated with *T. cruzi* include myocarditis caused by infiltration of inflammatory cells to sites such as the heart [269] and eventual parasite-induced autoimmunity [270].

T. brucei is an extracellular parasite transmitted to humans by tsetse fly. This parasite is able to evade the host immune response by constantly changing its Variable Surface Glycoproteins (VSG) surface coat, allowing it to establish and persist in the host [271,272]. *T. brucei* proliferates in the blood, and tissues of infected hosts, causing infiltration of inflammatory cells, and vasculitis [273]. In the latter stages of *T. brucei* infection, the parasite crosses the blood brain barrier and contributes to cerebral trypanosomiasis [271]. Similar to *T. cruzi, T. brucei* can modulate host immune responses and has numerous evasion strategies (reviewed in [272,274,275]).

2.3.2 Fish trypanosomes (*Trypanosoma carassii*)

Trypanosoma carassii (syn. *T. danilewskyi)* is an extracellular protozoan parasite infecting piscine hosts such as carp, goldfish, tench and eel [276]. *T. carassii* is similar to *T. brucei* as it is extracellular in nature, however it contains sialic acids like the intracellular *T. cruzi* [257]. Similar to mammalian trypanosomes, *T. carassii* is transmitted by a vector, in this case through a blood meal of a leech. In natural settings, infection with *T. carassii* is not considered pathogenic, however, in aquaculture setting, prevalence on infection can reach up to 100% causing significant mortality and morbidity [10,257]. Severity and mortality of infection is dependent on the initial numbers of parasites that enter the host [277]. Parasites replicate in the blood of fish, and reach peak parasitemia at approximately 2-3 weeks post infection [278]. Fish infected with *T. carassii* display symptoms of anemia and anorexia, and histopathalogical changes in organs can be observed [12,257,276].

Goldfish, which survive a primary infection with *T. carassii*, develop resistance to re-infection, indicating production of parasite-specific antibodies and apparent immunological memory. For example, immunization with serum or IgM from recovered hosts conferred protection in naïve hosts [279,280]. Moreover, immunization with *T. carassii* excretory/secretory (ES) products resulted in increased resistance, as shown by lower parasitemia [281]. Analysis of ES via mass spectroscopy revealed presence of tubulin (α and β) subunits, which were able to inhibit growth of *T. carassii in vitro* [282]. Immunization of goldfish with β-tubulin conferred partial resistance to *T. carassii* infections [283]. These data suggest that parasite-specific antibodies contribute to control

and elimination of *T. carassii.* However, researchers do not share the stance on the presence of immunological memory in teleosts. In mammals, long-lived logarithmic increases in antibody affinity are observed after infection or immunization [284]. Contrary to, teleosts display a modest increase in antibody response, and antibody heterogeneity is low [72]. For example, multiple immunizations are required for the detection of specific antibody titres [285], however this memory remains to be limited in time [286]. *T. carassii* has been shown to aggregate in internal organs such as the kidney or spleen [279,287,288]. Sequestration of parasites in internal organs may contribute to long-lasting resistance to re-infection due to the constant priming of the immune system [277,280].

Even so, *T. carassii* can maintain prolonged infections in fish, and the mechanisms behind its persistence remain unclear [257]. During the acute phase of infection, polyclonal activation of lymphocytes may contribute to immunosuppression of the host. For example, IgM purified from non-infected fish had higher trypanocidal activity then IgM purified from *T. carassii* during the acute stage of infection, suggesting *T. carassii* induced production of non-specific polyclonal antibodies, resulting in a dilution of specific IgM [289]. Additionally, *T. carassii* has numerous evasion strategies that may contribute to its persistence, which will be discussed in the subsequent section.

2.3.3 Immune evasion mechanisms of flagellates

Flagellates can modulate host immune responses by influencing cytokine production, and thus the effectiveness of host defense against intracellular or extracellular parasites. Th1-biased responses result in production of pro-inflammatory cytokines such as IFNγ, and TNFα, that result in macrophage and CTL activation and are important in elimination of intracellular pathogens [290,291]. Induction of Th2-biased responses is necessary for elimination of extracellular parasites by eosinophils and mast cells and is characterized by induction of cytokines such as IL-4 and IL-10 [290,291]. For example, a Th1 response is necessary for immunity against *Leishmania* spp [292]. However, *Leishmania major* can prevent its clearance by inducing IL-10 production by T regulatory cells [293]. Similarly, in *T. cruzi* infected mice elevated levels of IL-10 have been

documented and may contribute to parasite persistence [294]. Furthermore, increased IL-10 and TGFβ were associated with increased susceptibility of mice [295,296], which was also achieved by the blockade of IFNγ and TNFα in *T. cruzi* infected mice [297]. Therefore, the induction of IFNγ is required to activate phagocytes to destroy internalized parasites via NO production [298], as well as induction of TNFα that mediates resistance by inducing NO production against *T. cruzi* [299].

Contrary to intracellular pathogens such as *Leishmania* and *T.cruzi,* IL-4 is necessary for antibody production and controlling *T. brucei* infections in mice [300]. Moreover, *T. brucei* production of the trypanokine, trypanosome lymphocyte trigger factor (TLTF) induces production of IFNγ and TGFβ [301]. During acute stage of *T. brucei* infection, a higher induction of IFNγ and TNFα have been documented in mice, which favors a Th1-type immune response against intracellular pathogens [302], thereby allowing *T. brucei* to establish.

Sharing characteristics of both intra- and extracellular trypanosomes, *T. carassii* induced a mixed Th1/Th2 response in infected goldfish, with up-regulation of genes encoding for both pro- and anti-inflammatory cytokines [303]. In carp infected with *T. carassii,* an induction of IFNγ and IL-23 was observed, suggestive of a Th17-like immune response [304]. Moreover, *T. carassii* heat shock protein 70 (Tcahsp70) was identified using 2-dimensional gel electrophoresis, and shown to enhance pro-inflammatory responses in goldfish macrophages [305]. Although a mixed Th1/Th2 responses were observed, this finding suggests *T. carassii* may preferentially induce a Th1 immune response in goldfish to evade elimination via Th2 immune responses [257,305].

Trypanosomes have developed numerous evasion strategies. For example, *T. brucei* constantly modifies its VSG to evade host immune system, whereas *T. cruzi* has a sialic acid rich surface coat, and is intracellular to avoid detection. Similar to *T.cruzi, T. carassii* has mucin-like glycoproteins on the surface that are GPI-linked and modified with sugars such as sialic acids [306]. The presence of sialic acids contributes to a net negative charge, and provides protection against antibodies [307]. Additionally, these

mucin glycoproteins aid in the establishment of infection since they provide protection against the alternative complement pathways [306]. Parasites can dampen host immune responses by masking antigens using parasite trans-sialidase (TS) enzyme to transfer sialic acids from host glycococonjugates onto mucins of the parasite [265].

Both alternative and classical complement pathways are activated during infections with trypanosomes. Although *T. brucei* activates alternative pathway of complement, it is able to escape lysis via its VSG coat to prevent the association of C3 with parasite surface [308–310]. Furthermore, *T. brucei* glycoprotein 63 (gp63) impairs protein processing functions and therefore protects against complement-mediated lysis [311]. In *T. carassii,* a gp63 homologue was identified (Tcagp63) and may interfere with signal transduction as indicated by an attenuation of reactive oxygen and nitric oxide intermediate production [312]. Trypsinized *T. carassii* is susceptible to the alternative pathway of complement [313]. However, molecules expressed on parasite surfaces such as calreticulin (CRT), an E/S molecule, participates in modulation of the complement system of hosts [314,315]. For example, *T. cruzi* CRT inhibits the classical complement pathway [316]. Similarly, *T. carassii* calreticulin (TcaCRT) has been identified and documented to bind C1q inhibiting classical pathway of complement [9]. TcaCRT may play a protective role for *T. carassii* since complement lysis of trypsinized *T. carassii* has been demonstrated [313].

2.4 Trypanosomes and acute phase proteins

Trypanosomes infections have been documented to alter the expression of APPs in different experimental models. For example, mice infected with *T. brucei* displayed increased expression of serum amyloid P and haptoglobin [317]. Similar to, rabbits infected with *Trypanosoma evansi* displayed elevated levels of haptoglobin, however this increased was only observed in the chronic phase of infection [318].

Haptoglobin binds hemoglobin facilitating its rapid clearance and preventing heme-mediated oxidative damage. Trypanosomes have evolved mechanisms to acquire

iron components, such as high affinity heme binding proteins and receptors on their cell surfaces [319,320]. For example, *T. brucei* contains a haptoglobin-hemoglobin receptor within its VSG for acquisition of iron components [321]. Interestingly, in humans and mice, haptoglobin-related proteins (Hpr) such as trypanosome lytic factor-1 (TLF-1) when taken up by trypanosomes as they acquire iron, can directly kill them via oxidative damage [322,323]. Recent research has also demonstrated human Hpr was able to divert the function of *T. brucei* receptor-mediated uptake of iron components and mediate innate immune responses against *T. brucei* in humans [320]. Similarly, in *T. cruzi* infections haptoglobin affects the pathogenesis of disease. For example, Hp1-1 isoform confers protection against *T. cruzi* due to a rapid metabolism of haptoglobin-hemoglobin complexes and higher antioxidant activity [325,326].

Continuing on the topic of iron, trypanosomes require host transferrin to cover its iron needs [327] which is mediated through trypanosome expression of transferrin receptors [328]. *T. brucei* is able to switch between different transferrin-receptor genes, therefore overcoming transferrin host diversity [329]. During infections, mammalian hosts down-regulate expression of transferrin to limit iron availability [191], however trypanosomes can combat this by up-regulating their own receptor expression [330]. Nanobodies (monovalent antigen binding fragments) against *T. brucei* VSG were able to block transferrin uptake and contributed to trypanosome killing [331], emphasizing the importance of transferrin in trypanosome infections.

In mice infected with *Trypanosoma congolense* ceruloplasmin was up-regulated [332]. Similarly, *T. brucei* infected mice [333] and *T. evansi* infected cats [334], displayed higher expression of ceruloplasmin and were correlated to serum copper levels [334]. Ceruloplasmin has anti-oxidant and anti-inflammatory properties [89], however the exact role of ceruloplasmin in trypanosome infections is unclear i.e. where it causes iron influx or efflux from cells [92].

In other protozoan infections such as *Plasmodium falciparum,* α-1-acid glycoprotein inhibited parasite multiplication by blocking parasite-erythrocyte interaction [335]. Although α-1-acid glycoprotein is significantly up-regulated in *T. congolense* mice

[332] and *T. brucei* infected humans [336], it may not be play important roles in immunomodulation. For example, *T. cruzi* neuraminidase was able to hydrolyze α-1-acid glycoprotein with high affinity [337].

C-reactive protein and Serum amyloid P are up-regulated in several trypanosome infections. For example, up-regulation is observed in *T. congolense* infected mice [332], *T. brucei* infected dogs [338], and in *T. brucei* [336] and *T. cruzi* [339] infected humans. Despite the presence of phosphocholine on *T. brucei,* CRP-deficient mice were not more susceptible to trypanosome infection [96]. In *T. cruzi* infections elevated CRP levels were correlated to myocardial injury progression, suggesting it may be a marker of Chaga's Disease progression [339]. In addition to being a diagnostic tool, CRP plays a role in immunomodulation such as activating complement. However, trypanosomes have evolved mechanisms to disrupt the progression of these pathways. For example, *T. cruzi* produces complement regulatory protein, which is similar to the complement regulatory protein DAF as it is able to inhibit formation of C3 convertases [340]. Additionally, *T. cruzi* complement C2 receptor inhibitor traspanning (CRIT) interacts with complement component C2 to inhibit formation of C3 convertase [341].

Complement factor 3 is a key regulator of complement activation and binding to parasite surfaces contributes to opsonization and phagocytosis. Component C3 was up-regulated in carp in response to *T. borreli* infection [153], ectoparasite *Ichthyophthirius multifilis* infection [342], and detected in rainbow trout [2]. Contrary to, in animal trypanosomiasis, there was a negative correlation between complement components and parasites, suggesting trypanosomes themselves affect complement levels e.g. inhibiting formation of C3 convertase [308,343]. *T. carassii* can also inhibit complement activation through secretion of CRT which binds the C1q preventing downstream activation of the classical pathway of complement [9]. Similarly, *T. cruzi* CRT can bind C1q and MBL to inhibit classical and lectin complement pathways [314,341,344].

α-2-macroglobulin seems to have species dependent activities as an acute phase protein. For example in squirrels and rats it is considered a major APP, unlike other mammals [2,345]. In *T. cruzi* infection, mice displaying elevated levels of α-2-macroglobulin were more resistant to acute infection [346]. α-2-macroglobulin can

inhibit *T. cruzi* cysteine proteases such as cruzipain, and the ability to invade host cells [347–349]. Similar to, in rainbow trout α-2-macroglobulin inhibited *Cryptobia salmositica* proteases [350]. Up-regulation of α-2-macroglobulin was observed in grass carp infected with the parasitic copepod *Sinergasilis major* [220], and carp infected with *Trypanoplasma borreli* [153]. Homologues of acute phase proteins have been identified in numerous fish species and research elucidating expression of these proteins has been observed in response to chemical mimics, bacterial and viral infections however, limited information exists about the acute phase response to piscine trypanosomes.

From the above, it is clear that the acute phase response has been studied extensively in higher vertebrates such as humans and other mammals, however the characteristics of piscine APPs are not fully elucidated. Understanding the pathophysiology of the APR and the function and kinetics of APPs will provide a basis for future work in fish diseases. For example, APP could be used for clinical diagnosis and prognosis of infections in aquaculture. In addition, APPs can be used in environmental monitoring to assess fish welfare and evaluate immunotoxicity. Lastly, APPs have the potential to be used as markers of disease resistance, thereby providing long-term control over infection problems.

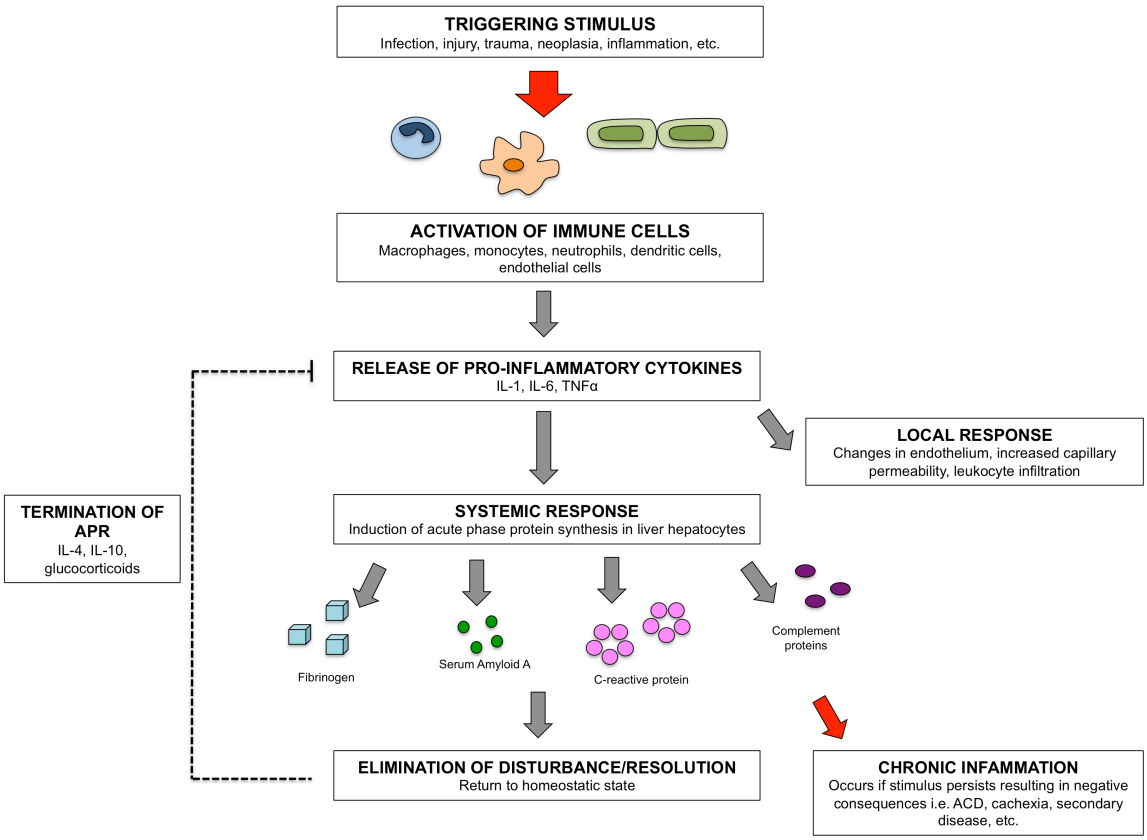

Figure 2.1 The initiation, progression and termination of the acute phase response. This figure reflects information drawn and adapted from [76,80,90,351].

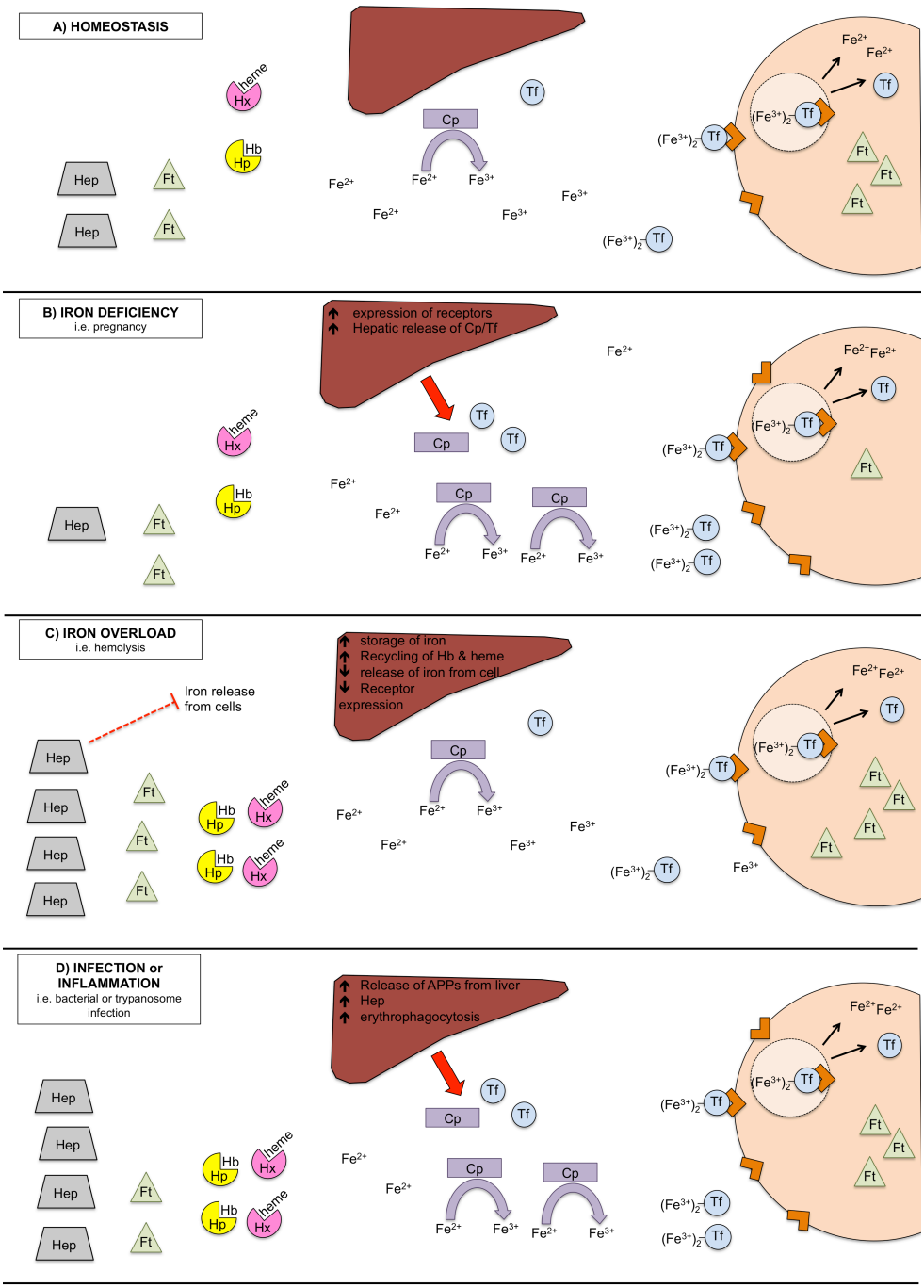

Figure 2.2 Simplistic overview of iron homeostasis in (A) Normal physiological conditions: Cp oxides Fe^{2+} to Fe^{3+}. Tf carries Fe^{3+} to Tf receptor on target organs, and is taken up via clatherin-dependent endocytosis. Within the lysosome, Fe^{3+} is reduced to Fe^{2+}. Hp binds Hb while Hx binds heme for recycling within the liver. **(B) In states of iron deficiency:** Hepatic synthesis and secretion of Tf and Cp occur. Additionally, expression of Tf receptors is increased to accommodate for decreased iron stores. Decreased Hep results in iron release from storage sites. **(C) in iron overload:** Increased Hep results in inhibition of iron release. Increased Hp and Hx prevents against oxidative damage. Decreased expression of Ft and Tf receptors protects from iron accumulation within organs and cells **(D) during infection or inflammation:** Hepatic synthesis and secretion of Tf and Cp occur. Increased Hp and Hx prevents against oxidative damage. This figure reflects information drawn and adapted from [160,190,199,352–354].

Table 2.1 Summary of classifications and biological functions of acute phase proteins. Acute phase proteins have been classified according to function, direction of change (positive or negative), amplification intensity (minor, intermediate or major) and according to cytokine regulation (Type I or II).

	Direction of change	Intensity	Cytokine regulation	Function
Major Acute Phase Proteins				
C-reactive Protein	Positive	Species dependent	Type I	Pentraxin recognizing phosphocholine; opsonin mediating phagocytosis & complement activation; induces expression of cytokines [103,107–109]
Serum Amyloid P	Positive	Species dependent	Type I	Pentraxin recognizing phosphoethanolamine [103]
Serum Amyloid A	Positive	Major	Type I	Immunomodulation (induction of pro- & anti-inflammatory cytokines; chemotactic recruitment of immune cells) [128,129,138,141,148]
Iron transport and metabolism				
Transferrin	Species dependent	-	-	Iron delivery; associated with innate responses (antimicrobial effector functions) [158,165–167]
Ceruloplasmin	Positive	Minor	Type II	Serum ferroxidase essential in iron homeostasis [168]
Haptoglobin	Positive	Species dependent	Type I/II	Binds hemoglobin with high affinity [179]
Hemopexin	Positive	Minor	Type I	Binds excess heme; antioxidant properties [190]
Ferritin	Positive	Intermediate	Type II	Involved in iron storage [193]
Hepcidin	Species dependent	-	Type II	Main regulator of iron homeostasis; antimicrobial peptide [199]
Protease Inhibitors				
α-1-antitrypsin	Positive	Intermediate	Type II	Serine protease inhibitor; inhibits neutrophil elastase and proteinase 3; inhibits TNFα and superoxide production in neutrophils [204,206]
α-1-anti-chymotrypsin	Positive	Intermediate	Type II	Serine protease inhibitor; inhibits capthesin G [211]
α-2-macroglobulin	Species dependent	Species dependent	Type II	Inhibits parasitic/pathogenic proteases [212,213]
Coagulation Factors				
Fibrinogen	Positive	Intermediate	Type II	Important in coagulation & wound healing; regulator of inflammatory responses (activation & recruitment of immune cells) [223,224]
Plasminogen	Positive	Intermediate	-	Precursor to serine protease, plasmin important in control of inflammation & clotting [226]
Complement Proteins				
C3	Positive	Minor	Type I	Key component in complement pathways [238]
C3a, C5a	Positive	Minor	-	Anaphylatoxins [233,234]
C5b-C9	Positive	Minor	-	Forms the membrane attack complex [231]
Other Acute Phase Proteins				
α-1-acid glycoprotein	Positive	Major	Type I	Binds microbes & drugs; anti-inflammatory properties [245,246]
LPS binding protein	Positive	Species dependent	Type I	Binds LPS; modulates innate immune responses [250]

CHAPTER 3

MATERIALS AND METHODS

3.0 General Procedures

3.1 Fish and maintenance of the parasite

3.1.1 Fish

Goldfish (*Carassius auratus* L.) (3-6 cm in length) were purchased from Aquatic Imports (Calgary, Alberta, Canada) and maintained in the Aquatics Facility in the Biological Sciences Building at the University of Alberta. Fish were kept in a continuous flow-through water system at 20°C, on a simulated natural photoperiod (14 hour light, 10 hour dark), and fed trout pellets daily until satiated. Fish were acclimated for at least three weeks prior to use in experiments. Prior to handling or manipulation, fish were sedated with tricaine methane sulfonate (TMS; 40 mg/L). The care of experimental animals followed guidelines of the Canadian Council of Animal Care (CCAC-Canada).

3.1.2 Collection and preparation of fish serum

Goldfish blood was obtained by bleeding anaesthetized fish from the caudal vein using a 23-guage or 25-guage needle fitted syringe. After allowing blood to clot overnight at 4°C overnight, serum was isolated by centrifugation at 1540 x *g* for 30 minutes. Serum used for maintenance of parasite cultures was heat-inactivated at 56°C for 30 minutes, filter-sterilized (Millipore, 0.22μm) and frozen at -20°C until used. Carp serum (*Cyprinus caprio*) was collected and prepared using the same procedures as those for goldfish and used in cultivation and maintenance of goldfish cells. Immune serum was collected from individual fish infected with 6.25×10^6 or 1.0×10^7 *in vitro* grown parasites. Sera were collected on 3,7,14, 21, 28 and 56 days post-infection, and stored at -20^{o}C until used.

3.1.3 Parasites

Trypanosoma carassii strain TrCa (syn. *T. danilewskyi*) was used in this study. Parasites were cultured *in vitro* in TDL-15 medium (Table 3.1) supplemented with 10% heat-inactivated goldfish serum at 20°C as previously described [278,355]. Trypanosomes used for all assays were obtained from 5-7 day old stock cultures.

3.1.4 Purification of parasites from blood

Trypanosomes were purified from the blood as previously described [288]. Briefly, tri-sodium sodium citrate (100 mM sodium citrate, 40 mM glucose, pH 7.3) was added to blood collected from infected fish to prevent clotting, after which it was centrifuged at 750 *x* g for 10 minutes and the supernatant removed. The layer on top of the red blood cell pellet containing trypanosomes was removed and gently re-suspended in suspension buffer (57 mM Na_2HPO_4, 3 mM $NaHPO_4$, 44 mM NaCl, 4 mM KCl, 5 mM glucose, 80 mM sucrose, pH 8). The re-suspension step was repeated twice to ensure most of the blood cells were removed. Following the last centrifugation step, the vial was titled at a 20° angle for 5 minutes to allow parasites to separate from the pelleted cells. The separated parasites were removed and suspended in culture medium.

3.1.5 Infection of fish

Fish were infected intraperitoneally with 6.25×10^6 or 1×10^7 *in vitro* grown parasites suspended in 100 µL of TDL-15 medium, or sham injected with 100 µL of TDL-15 (control) using a 1 mL tuberculin syringe fitted with a 25-guage needle.

3.1.6 Assessment of infection (parasitemia)

Fifty microliters of blood was removed on days 3, 7, 14, 21, 28, and 56 post-infection and 4 µL of undiluted blood was added to 96 µL of tri-sodium citrate anticoagulant solution. Parasitemia was determined using the methods described previously [281,355]. Briefly, the number of trypanosomes per ml of blood was determined using an improved Neubauer hemocytometer fitted with a glass cover slip (22 x 22 mm) and a bright field microscope (400x).

For fish with very low circulating parasites that were not detectable using the haemocytometer, blood collected in a heparinized capillary tube was examined for the presence of parasites using the hematocrit centrifugation technique [356].

3.1.7 Effects of goldfish complement on *T. carassii in vitro*

3.1.7.1 Enzymes and inhibitors

Trypsin (TPCK-treated) and Soybean trypsin inhibitor (SBTI) were purchased from Sigma. A 1 mg/mL trypsin and SBTI solution was prepared in serum-free medium and used at a concentration of 0.1 mg/mL

3.1.7.2 Enzyme treatment of trypanosomes

Trypanosomes cultured *in vitro* were washed twice in serum-free medium and re-suspended to 1 x 10^6/mL. Medium alone or 0.1 mg/mL trypsin (Trypsin (TPCK-treated), Sigma) treatments were added to 1 mL of trypanosome suspension and incubated for 1 hour at 20°C. Subsequently, 10% HI-GFS or 0.1 mg/mL Soybean trypsin inhibitior:SBTI (Sigma) was added to the reaction tubes and the tubes incubated for 10 minutes at 20°C. After enzyme inactivation, trypanosomes were washed twice in serum-free medium and re-suspended in 1 mL of medium for use in the assay

3.1.7.3 Trypanosome lysis assay

The procedure to assess trypanosomes susceptibility to lysis was described previously [313]. Briefly, 1 x 10^5 parasites were seeded in a 96-well plate in either medium, non-immune, heat-inactivated (HI) non-immune or immune goldfish serum or normal goldfish serum supplemented with either PBS or 0.5 μg/mL or 5 μg/mL of recombinant goldfish C-reactive protein (rgfCRP). Plates were incubated for 1 hour at 20°C, after which the contents of each well were re-suspended in medium and viable trypanosomes enumerated using a hemocytometer. Trypanosomes were considered viable when flagellar movement was observed.

3.2 Isolation and cultivation of goldfish kidney leukocytes

3.2.1 Culture Medium

The culture medium used for cultivation of goldfish leukocytes has been previously described [357,358]. The composition of incomplete medium (MGFL-15) is shown in Table 3.2. The composition of medium constituents is shown in Table 3.3. Complete medium (C-MGFL-15) was composed of MGFL-15 containing 100 U/mL

penicillin/100 μg/mL streptomycin (Invitrogen), 100 μg/mL gentamycin (Gibco), 10% newborn calf serum (NCS: Hyclone, Loan, UT), and 5% carp serum.

3.2.2 Isolation and establishment of primary kidney macrophages (PKM)

The procedure for the isolation and cultivation of primary kidney macrophages (PKM) has been previously described [11]. Briefly, the kidney were aseptically removed from individual fish, and passed through stainless steel screens using incomplete medium containing antibiotics (100 U/mL penicillin/100 μg/mL streptomycin, Invitrogen), and heparin (50 U/mL, Sigma). The resulting cell suspensions were layered on 51% Percoll (Sigma) and centrifuged at 400 x *g* for 25 minutes. The cells at the Percoll-media interface were removed and washed twice in MGFL-15 (230 x *g* for 10 minutes).

PKM cultures were established by seeding freshly isolated PKMs into 75 cm^2 culture flasks containing 15 mL of complete media (C-MGFL-15) and 5 mL of cell-conditioned medium (CCM) from previous cultures. The PKM cultures consisted of a heterogeneous population of cells, which include early progenitors, monocytes, and mature macrophages. Day 3 to 4 cultures consisted primarily of monocytes whereas older cultures (6-7 days) consisted primarily of macrophages. In this thesis, day 3 PKM cultures are referred to as monocytes and day 6-7 PKMs are referred to as macrophages. Based on previously established parameters, PKM cultures were sorted into mature macrophage and monocyte populations based on internal complexity and size using the FacsAria flow cytometer (Becton Dickson) [11]. The number of viable cells was determined using the trypan blue staining.

3.2.3 Isolation and establishment of kidney-derived neutrophils

The procedure for isolation of kidney neutrophils has been previously described [60]. Briefly, kidneys were removed from individual fish and passed through steel screens, homogenates collected, and layered on a 51% Percoll solution, and centrifuged at 400 x *g* for 25 minutes. The pellet containing red blood cells and kidney-derived granulocytes were re-suspended in 1X red blood cell (RBC) lysis buffer (144 mM NH_4Cl, 17 mM Tris, pH 7.2) to lyse the red blood cells. The cells were washed twice in NMGFL-15 (230 x *g* for 10 minutes) and seeded into 25 cm^2 culture flasks containing 10% carp

supplemented MGFL-15. Non-adherent cells were harvested and washed twice in NMGFL-15 (230 x *g* for 10 minutes) prior to use.

3.2.4 Isolation of splenocytes

The procedure for the isolation of splenocytes has been previously described [65]. Briefly, spleens were removed from individual fish and passed through steel screens using incomplete medium containing antibiotics (100 U/mL penicillin/100 μg/mL streptomycin, Invitrogen). The cell suspensions were then layered on a 51% Percoll solution, and centrifuged at 400 x *g* for 25 minutes, after which the 51% Percoll-medium interface was removed and washed twice with MGFL-15 (230 x *g* for 10 minutes).

3.2.5 Isolation of peripheral blood leukocytes (PBLs)

The procedure for the isolation of PBLs has been previously described [65]. Blood was collected from the caudal vein of individual fish using heparinized needles and suspended in NMGFL-15 containing 100 U/mL penicillin/100 g/mL streptomycin. PBLs were pelleted by centrifugation at 400 x *g* for 10 minutes after which the red blood cells were lysed using 1X RBC lysis buffer for 30-40 minutes on ice. The sample was washed twice in NMGFL-15 (230 x *g* for 10 minutes) to collect PBLs.

3.2.6 Preparation of cell-conditioned medium

Cultures of primary kidney macrophages maintained for 6-8 days at 20°C were centrifuged at 400 x *g* for 10 minutes, and the supernatant (CCM) was collected from individual cultures when total cell counts exceeded 10 million cells/individual culture, pooled, filter-sterilized (0.22 μm) and stored at 4°C until further use.

3.3 Molecular procedures

3.3.1 Cloning of *C. auratus* genes

To clone CRP and SAA, primers were designed based on the conserved nucleotide sequences against the CRP gene of common carp (Accession No. AB028455.1) and zebrafish (Accession No. JF772178.1) and against the SAA gene of common carp (Accession No. AB016524.1) and zebrafish (Accession No.

NM_001005599.1). RACE PCR was performed using the SMART RACE PCR (Clonetech, USA) to obtain the open reading frame and untranslated regions of the transcript. The primers used in homology based PCR, RACE-PCR primers can be found in Table 3.5.

3.3.2 *In silico* analysis of *C. auratus genes*

Protein alignments were performed using Clustal W or Omega software. Conserved regions and motifs identified by Pfam (http://pfam.sanger.ac.uk/search/sequence). Phylogenetic analysis was done using MEGA5 software neighbor joining method and bootstrapped 10,000 times [359]. The full-length sequence for CRP (Accession No. KP057691).and SAA (Accession No. KT598223) have been submitted to Genbank.

3.4 Prokaryotic expression of *C. auratus* recombinant proteins

3.4.1 Cloning of *C. auratus* genes into pET SUMO expression vector

PCR fragment encoding the full sequence of goldfish CRP and SAA ORF was amplified using primers that allow cloning into pET SUMO expression vector (Invitrogen). PCR products were gel purified (QIAquick Gel Extraction Kit, Qiagen) and ligated into pET SUMO vector (Invitrogen). The recombinant plasmid was transformed into competent *Escherichia coli* (NEB10, Biolabs) and plated onto LB-kanamycin plates (50 µg/mL) and incubated at 37°C overnight. Positive recombinant clones were identified by colony PCR, cultured in LB-kanamycin, and plasmid DNA purified from recombinant clones using QIAprep spin Miniprepkit (Qiagen). To verify that the insert was in the correction orientation and frame, the purified plasmids were sequenced using vector specific primers. The pET SUMO vector encodes an N-terminal 6XHis tag for purification and detection of the recombinant molecules. All primers used in the generation of recombinant protein expression constructs can be found in Table 3.6.

3.4.2 Pilot expression of recombinant CRP and SAA

Recombinant plasmids containing CRP or SAA inserts were transformed into BL21 Star One Shot *E. coli* cells (Invitrogen). In this pilot study, 1 mL of transformation mixture was used to inoculate 10 mL LB-kanamycin (50 µg/mL), which was grown to

mid-log phase (OD_{600} of 0.5-0.6) and split into five 1 mL cultures. IPTG (0.25 mM, 0.5 mM, 1 mM) was added to induce expression of recombinant proteins for 3 hours. Bacterial lysate supernatant and pellet were resolved by SDS-PAGE and visualized by Western blot using mouse monoclonal antibody (1:5000) against the N-terminus 6X His tag. From this pilot study, I determined that the recombinant CRP was present in the pellet whereas SAA was present in the pellet and to a lesser extend in the supernatant, and the optimal induction concentration was 1 mM IPTG.

3.4.3 Scale up production of recombinant goldfish CRP and SAA in *E. coli*

Recombinant plasmids containing CRP or SAA inserts were transformed into BL21 Star One Shot *E. coli* cells (Invitrogen), scaled up to mid-log phase (OD_{600} of 0.4-0.6), and induced with 1 mM IPTG for 3 hours. Optimal induction time and concentration was determined in a pilot study. The induced bacteria was pelleted and frozen at -20°C until needed. The bacteria re-suspended in lysis buffer (2.5 mL of 10X FastBreak Lysis Reagent (Promega) in 22.5 denaturing wash buffer (100 mM Hepes, 10 mM imidazole, 7.5 M urea, pH 7.5)) and incubated with MagneHis Ni-particles (Promega) as recommended by manufacturer. A PolyATtrack System 1000 magnet (Promega) was used to retain Ni-particles bound to recombinant proteins. Protease Inhibitor cocktail (500 μM AEBSF, HCl, 150 nM Aprotonin, 1 μM E-64, and 1 μM Leupeptin) was added to all buffers to prevent protein degradation. The supernatants were discarded, the beads washed 3 times under denaturing conditions (as described above), and the recombinant proteins eluted from the beads using 500 mM imidazole. To confirm the presence of recombinants, a western blot was performed using the anti-6X His tag antibody. Subsequently, the protein was renatured overnight in 10 volumes of renaturation buffer (4 mM reduced glutathione, 2 mM oxidized glutathione, 50 mM sodium borate, 5 mM EDTA, pH 8.5), and dialyzed overnight against 4 L of 1X PBS overnight using 20 kDA MWCO Snakeskin dialysis tubing (Pierce). To concentrate protein, the dialysis tubing was placed in polyethylene glycol flakes for 8 hours and further dialyzed against PBS overnight to remove traces of imidazole and urea. The recombinant protein was passed through Pierce High Capacity Endotoxin Columns (Thermo Scientific) according to manufacturers directions. Recombinant protein was analyzed using the *Limulus* amebocyte lysate kit (Thermo Scientific) and found to be endotoxin free (<0.1 EU/mL).

Protein concentration was determined using a Micro BCA Protein Assay Kit (Thermo Scientific). The presence of recombinant protein was confirmed by Western blot and the identity of the protein confirmed using mass spectrometry.

3.5 Quantitative expression analysis (Q-PCR) of goldfish acute phase proteins

3.5.1 Quantitative PCR design and validation

Quantitative PCR (Q-PCR) primers were designed using Primer Express Software (Applied Biosystems). Primers were validated by assessing the relative quantitative efficiency against a curve designed from serial dilutions of goldfish cDNA. Primers were chosen by assessing R^2 value, y-intercept range, percentage efficiency, and visualizing melting curves for single peaks. To confirm primer specificity, Q-PCR products were run on a gel and sequenced. All primer sequences are shown in Table 3.7.

3.5.2 Quantitative PCR analysis of APP expression in non-stimulated goldfish immune populations

Cell populations including goldfish mature macrophages, monocytes, neutrophils, splenocytes, and PBLs were isolated (*n*=5) as described. Total RNA was isolated immediately after cell isolation using TRIzol Reagant (Invitrogen) and reverse transcribed into cDNA using SuperScript III cDNA kit (Invitrogen) according to the instructions by the manufacturer. Thermocycling parameters were as follows: 95ºC, 2 minutes; 95ºC, 15 seconds; 60ºC, 1 minute; 95ºC, 15 seconds; 60ºC, 1 minute; 95ºC, 30 seconds; 60ºC, 15 seconds (melting curve); for 40 cycles. Analysis of gene expression was performed using 7500 Fast Software (Applied Biosystems). Expression analysis of APPs was performed relative to the endogenous gene, elongation factor 1 alpha (EF-1α). The mean Ct value for EF-1α was in the range of 21.54 - 21.8 for all immune cell populations. The RQ values were normalized against the lowest expression of APPs (macrophages).

3.5.3 Quantitative PCR analysis of APP expression in normal goldfish tissue

Total RNA was isolated from kidney, liver, spleen, heart, brain, muscle, intestine and gill of healthy goldfish (*n*=6 for each tissue) using TRIzol reagent (Invitrogen) and

transcribed into cDNA using Superscript III cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. Quantitative expressions of APPs were performed using the 7500 Fast Real-Time PCR machine (Applied Biosciences). The cycling parameters were the same as described in Section 3.5.2. Expression analysis of APPs was performed relative to the endogenous gene, EF-1α. The mean Ct value for EF-1α was in the range of 19.34 - 19.76 for all tissues examined. The RQ values were normalized against the expression seen in the lowest tissue group (muscle).

3.5.4 Quantitative PCR analysis of APPs in non-infected and *T. carassii* infected goldfish

Kidney, liver and spleen were removed from non-infected control (*n*=6) and infected (*n*=6) goldfish on 3, 7, 14, 21 and 56 days post infection. Samples were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA processing.

Kidney, spleen, and liver RNA was extracted from tissues using TRIzol reagent (Invitrogen) or RNAeasy Mini Kit according to manufacturer's instructions. 2.5 µg of total RNA was reverse transcribed into cDNA using Superscript III cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. The cycling conditions are the same as described in Section 3.5.2. The fold difference (RQ) was found as instructed by the qPCR instruction manual (Applied Biosystems) in comparison to the endogenous control gene, EF-1α. All samples were run in triplicate and presented as the mean ± SEM of 6 fish per experimental group.

3.5.5 Quantitative PCR analysis of pro-inflammatory and anti-inflammatory cytokine expression in treated PKMs

Monocytes and macrophages were obtained from individual fish (*n*=4 for each) and seeded into individual wells of a 24-well plate at a density of 1 x 10^6 cells per well in a final volume of 500 µL CMGFL-15. Cells were treated with PBS, 0.5 µg/mL rgSAA, or 5 µg/mL rgSAA for 1, 3, 6, and 12 hours. Total RNA was isolated from cells at indicated times and reverse transcribed into cDNA as described in previous sections. Changes in expression of select immune genes (IL-1β1, IL-1β2, IL-10, IL-12p35, IL-12p40, NLRP3,

TGFβ, TNFα1, TNFα2 and SAA) were assessed. All primer sequences used in Q-PCR are shown in Table 3.7.

3.6 Measurement of functional responses in goldfish immune cells

3.6.1 Respiratory burst assay

Respiratory burst assay was performed as previously described [65]. Briefly, goldfish monocytes from three to four day old cultures from individual fish were seeded into 96-well plates at a density of 2 x 10^5 or 3 x 10^5 cells per well and incubated in culture medium alone (control) or treated with various concentration of recombinant APPs. The cell cultures were incubated for 16-18 hours at 20°C after which NBT (2 mg/mL, Sigma) and PMA (final triggering concentration 100 ng/mL, Sigma) in PBS were added to the cultures and incubated at room temperature for 30 minutes. The plates were centrifuged at 400 x *g* for 10 minutes, supernatants aspirated, and pelleted cells fixed with methanol. Non-reduced NBT was removed by washing twice with methanol and reduced NBT was dissolved by 2 M KOH. To induce a colorimetric response, DMSO was added and plates read at 630 nm. To factor in background NBT reduction, absorbance from cells alone (no PMA) was subtracted from treatment values.

3.6.2 Nitric oxide assay

Nitric oxide assay was performed as performed as previously described [65]. Briefly, macrophages from six to eight day cultures from individual fish were seeded in wells of 96-well plates at a density 2 x 10^5 or 3 x 10^5 cells per well. These were then incubated in culture medium alone (control) or treated with various concentration of recombinant APPs and incubated at 20°C for 48 to 72 hours. Nitrite production was determined using the Griess reaction. Briefly, 1% sulphanilamide and 0.1% N-napthyl-ethylenedamine were added to cultures and nitrite levels were determined colorimetrically at 540 nm using a nitrite standard curve.

3.6.3 Chemotaxis assay

Cells from either day 3 (monocytes) or days 6–7 (mature macrophage) cultures or neutrophils were washed twice in incomplete NMGFL-15 and adjusted to a final concentration of 1 x 10^6 cells/mL. The chemotaxis assay was performed using blind well

leucite chemotaxis chambers (Nucleoprobe Corp.). The top and bottom chambers were separated by a 5 μm pore size polycarbonate membrane filter (Neuroprobe). Negative controls consisted of medium alone and the positive control was 20 ng/mL fMLP (N-formyl-methionyl-leucyl-phenylalanine). The chemokinesis control consisted of equal amounts of the treatment in both the upper and lower chambers.

For chemotaxis experiments the incubation period was 4 hour at 20 °C for PKMs and 1 hour at 20 °C for neutrophils. Following incubation the cell suspensions were carefully aspirated from the top chamber and the filters fixed in methanol, stained with Gill's Solution 3 (Sigma), and applied bottom side up on a microscope slide. Determination of chemotactic activity was assessed by counting the total number of cells found on the underside of the polycarbonate filters in 20 random fields of view under oil immersion (100X). Separate PKM cultures were established from four individual fish.

3.6.4 Phagocytosis assay

3.6.4.1 Preparation of FITC labeled *T. carassii* and GFP-labelled *E. coli*

T. carassii was labelled with 500 ng/mL FITC with continuous shaking at room temperature for 30 minutes. After staining, *T. carassii* was washed twice with 1X PBS. A growth curve of *E.coli* DH5α-GFP was generated correlating to A_{600} in conjunction with the number of colony forming units (CFUs) after plating bacterial serial dilutions. *E.coli* DH5α-GFP was washed twice with 1X PBS before use in experiments

3.6.4.2 Phagocytosis assay using ImageStream

T. carassii-FITC and *E.coli* DH5α-GFP were opsonized with either: 0.5 and 5 μg/mL rgfCRP or a PBS control, after which targets were washed twice with 1×PBS to remove unbound particles. *E.coli* DH5α-GFP were added to 1 x 10^5 macrophages at a ratio of 5:1 (bacteria/parasite: cells), and incubated for 2 hours at 20°C. After incubation, the samples were fixed in 1% formaldehyde at 4°C for 20 minutes. Collection of data was done using the ImageStream multi-spectral imaging flow cytometer (Amnis) and analyzed using INSPIRE software. A minimum of 10,000 cells was acquired. Phagocytic index was calculated based on the number of particles/parasites internalized per macrophage.

3.6.4.3 Phagocytosis assay using Quanta SC

E.coli DH5α-GFP was opsonized with varying concentrations of rgSAA (0.01, 0.1, 1, and 10 μg/mL) or a PBS control for 30 minutes, after which it was washed twice with 1X PBS to remove unbound particles. The bacteria were added to cells (neutrophils and macrophages) at a ratio of 3:1 (bacteria:cells) and incubated for an additional 30 minutes for neutrophils an 1 hour for macrophages. After incubation, the samples were fixed in 1% PFA at 4°C for 20 minutes. Collection and analysis of data was done using the Cell Lab Quanta SC flow cytometer.

Mature macrophages or neutrophils were pre-treated with varying concentrations of rgSAA (0.01, 0.1, 1, and 10 μg/mL) or a PBS control for 30 minutes, after which *E.coli* DH5α-GFP was added at a ratio of 3:1 (bacteria:cells) and incubated for an additional 30 minutes for neutrophils an 1 hour for macrophages. After incubation, the samples were fixed in 1% PFA at 4°C for 20 minutes. Collection and analysis of data was done using the Cell Lab Quanta SC flow cytometer.

3.7 SDS-PAGE and Western blot

3.7.1 SDS-PAGE

Proteins were separated and visualized by reducing SDS-PAGE according to the method described originally by Laemmli [360]. Briefly, samples were dissolved in Laemmli sample buffer (BioRad) and heated at 95 °C for 10 minutes. Samples were electrophoresed through polyacrylamide gels (4% stacking, 12% separating) at 100 V for 15 minutes followed by 130 V for 1 hour. The polyacrylamide gels were stained with Coomassie brilliant blue (BioRad) to visualize protein bands in some experiments. Coomassie staining was performed by soaking the gels in a solution of Coomassie brilliant blue (0.1% Coomassie brilliant blue, 10% acetic acid, 40% methanol) for 1 hour at room temperature). Gels were destained using 10% acetic acid and 40% methanol overnight.

3.7.2 Western blot

Following gel electrophoresis, proteins were transferred to 0.2μm nitrocellulose membranes (BioRad) at 125 V for 50 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes containing transferred proteins were blotted in the following manner. The nitrocellulose were blocked with 5% Skim Milk in Tris-buffered saline/Tween 20 (TTBS; 0.1% Tween 20 in 100 mM Tris-HCl, 0.9% NaCl, pH 7.5; TBS) for 1 hour at room temperature or at 4°C overnight. Membranes were washed 3X in TTBS, then incubated in a solution containing primary antibody in 2.5 % blocking buffer or TTBS and incubated for 1-2 hours at room temperature or overnight at 4°C. Membranes were washed 3X in TTBS for 5 minutes each before incubation with secondary antibody for 1 hour at room temperature. Blots were washed 3 times in TTBS for 5 minutes each. Protein bands were visualized using chromogenic ECL development solution according manufacturer's instructions (Thermo Scientific).

3.8 Statistical analysis

All statistical analysis was performed using GraphPad Prism 6. Data for antimicrobial responses (respiratory burst, nitric oxide, chemotaxis) as well as quantitative expression analysis were analyzed using one-way ANOVA followed by post-hoc tests. Probability of $P < 0.05$ was considered significant.

Table 3.1 Components of *Trypanosoma carassii* TDL-15 Medium[a]

Reagants/Solutions	Amount (per 500 mL)	Specifications
Milli-Q Water	150 mL	Endotoxin-free
Hank's Solution	20 mL	10X, no Ca^{2+} or Mg^{2+}
MEM Amino Acid Solution[b]	6.25 mL	50X
MEM Non-essential Amino Acid Solution[b]	6.25 mL	100X
$NaHCO_3$	0.63 g	
NaOH	15 μL	
Sodium pyruvate solution[b]	6.25 mL	10 mM
MEM Vitamin Solution[b]	5 mL	100X
Nucleic Acid Precursor Solution[c]	5 mL	2.5 mM
L-glutamine Solution	5 mL	200 mM
Gentamicin[d]	0.5 mL	50 mg/mL
2-Mercapthoethanol	0.5 mL	50 mM (fresh)
HEPES	1 g	
Insulin	0.0025 g	
GFL-15 Medium[e]	250 mL	

pH to 7.2 and filter sterilize using a 0.2 μm filter. Store at 4°C

[a] Developed by Wang and Belosevic [278]

[b] Supplied by Invitrogen

[c] 2.5 mM each of: adenosine, cytidine, hypoxanthine, thymidine and uridine

[d] Supplied by Gibco

[e] Leibovitz-15 and Dulbecco's Modified Eagles Medium 50:50 (v:v)

Table 3.2 Components of MGFL-15

Reagants/Solutions	Amount (per 1 L)	Specifications
Hepes	3.5 g	
KH_2PO_4	0.344 g	
K_2HPO_4	0.285 g	
NaOH	0.375 g	
$NaHCO_3$	0.17 g	
L-glutamine	0.2922 g	
Insulin	0.005 g	
10X Hanks Balanced Salt Solution	40 mL	
MEM Amino Acid Solution[a]	12.5 mL	50X
MEM Non-essential Amino Acid Solution[a]	12.5 mL	100X
MEM Sodium Pyruvate Solution[a]	12.5 mL	100X
MEM Vitamin Solution[a]	10 mL	
Nucleic Acid Precursor Solution[c]	10 mL	
2-Mercapthoethanol	3.5 µL	
GFL-15	500 mL	
Milli-Q water	Fill to 1 L	Endotoxin-free

pH to 7.4 and filter sterilize using a 0.2 µm filter. Store at 4°C

[a] Supplied by Invitrogen

[b] GFL-15 medium contains 1 package of dry powder of Leibovitz-15 and 1 package of dry powder of Dulbecco's Modified Eagle Medium in 2 L

[c]Nucleic Acid Precursor Solution is composed of 0.067 g adenosine, 0.061 g cytidine, 0.034 g hypoxanthine, 0.061 g thymidine, and 0.061 g uridine in 100 mL Milli-Q water.

Table 3.3 Constituents of Hank's Balanced Salt Solution

Reagants/Solutions	Amount (g per 500 mL)
KCl	2.0
KH_2PO_4	0.30
NaCl	40.0
$Na_2HPO_4*7H_2O$	0.45
D-Glucose	5.0
Phenol Red	0.05

Filter sterilized using a 0.2 μm filter. Store at 4°C

Table 3.4 Constituents of Nucleic Acid Precursor Solution

Reagants/Solutions	Amount (g per 100 mL)
Adenosine	0.067
Cytidine	0.061
Hypoxanthine	0.034
Thymidine	0.061
Uridine	0.061

Dissolved at 50°C water bath. Do not filter.

Table 3.5 Constituents of Phosphate Buffered Saline (PBS)

Reagants/Solutions	Amount (g per 1 L)
NaCl	8
KCl	0.2
$Na_2HPO_4*7H_2O$	2.68
KH_2PO_4	0.24

pH to 7.4 and filter sterilize using a 0.2 μm filter.

Table 3.6 Primers used for molecular cloning of *C. auratus* Serum amyloid A (SAA) and C-reactive protein (CRP)

Primer Name	Sequence (5'-3')
Homology Based Primers	
CRP forward	TTTACTCTCTGCATGCGTGTGG
CRP reverse	ATCAGGGTCTTGTCCGAGCAC
SAA forward	TGCACGTGGGAACTATGATGCT
SAA reverse	TTCTGTTGCAGTGATGCTCTTCTT
RACE PCR primers	
CRP 3' race	TCTGCATGCGTGTGGCGACG
CRP 3' nested race	ACCGCACGCCCGAGTTTGAT
CRP 5' race	ACGGTGCCACCAGGACGGAT
CRP 5' nested race	AACTCGGGCGTGCGGTAAGC
SAA 3' race	ATTACGTCCCCAGCGGTTGGC
SAA 3' nested race	TTACCGCGGCGAGAGAGTCC
SAA 5' race	AGAAGGGGCCCTGGAGGCAAA
SAA 5' nested race	TGCAGGGACTCTCTCGCCGC
Prokaryote Expression Primers	
CRP forward	ATTACTGAAGTGGGCCTCAGTG
CRP reverse	GTGCTAGTGGTGCAAATTAACTGA
SAA forward	CAATGGCACCGCTACCCAG
SAA reverse	TCAGTACTTTCTGGGAAGGCCC

Table 3.7 List of quantitative PCR primers

Primer	Sequence (5'-3')
CRP forward	GAT GGT TGT GTG GCC TTG GT
CRP reverse	AGG CAG ATG GAA AAA TGC TTC T
Cp forward	GCC ACT TCC TGC TGC ATT G
Cp reverse	TCC TTA GGA ACC ACC CAC TCA
EF-1α forward	CCG TTG AGA TGC ACC ATG AGT
EF-1α reverse	TTG ACA GAC ACG TTC TTC ACG TT
Hx forward	CATCACCTGGGACATGTGGAT
Hx reverse	GGTGTTCTGGGCTGTCTTCAG
IL-1β-1 forward	GCG CTG CTC AAC TTC ATC TTG
IL-1β-1 reverse	GTG ACA CAT TAA GCG GCT TCA C
IL-1β-2 forward	GAT GCG CTG CTC AGC TTC T
IL-1β-2 reverse	AGT GGG TGC TAC ATT AAC CAT ACG
IL-10 forward	CAA GGA GCT CCG TTC TGC AT
IL-10 reverse	TCG AGT AAT GGT GCC AAG TCA TCA
IL-12 p35 forward	TGT TTT ACG TGC ATT CCT TTG G
IL-12 p35 reverse	GGC GCC TGA AAA AAA TAC GA
IL-12 p40 forward	CTT CAG AAG CAG CTT TGT TGT TG
IL-12 p40 reverse	CAG TTT TTG AGA GCT CACCGA TAT C
NLRP3 forward	CAG AAG ACG CTC TCG GGT ACA
NLRP3 reverse	TCA GCT CCC AGT ATG CCA ATT
Tf forward	AGC CAT ATG CGG CCA AAC
Tf reverse	AGG AGC GCA GCC TTT ACT GA
TGFβ forward	GTA CAC TAC GGC GGA GGA TTG
TGFβ reverse	CGC TTC GAT TCG CTT TCT CT
TNFα1 forward	CAT TCC TAC GGA TGG CAT TTA CTT
TNFα1 reverse	CCT CAG GAA TGT CAG TCT TGC AT
TNFα2 forward	TCA TTC CTT ACG ACG GCA TTT
TNFα2 reverse	CAG TCA CGT CAG CCT TGC AG
SAA forward	TCT CTC GCC GCG GTA ATT C
SAA reverse	GGA AGG CCC TTG GGT CTG TA

CRP = C-reactive Protein; Cp = Ceruloplasmin; EF-1α = Elongation Factor-1-alpha; Hx= Hemopexin; IL-1β-1 = Interleukin-1beta-1; IL-1β-2 = Interleukin-1beta-2; IL-10 = Interleukin-10; IL-12p35 = Interleukin 12 p35; IL-12p40 = Interleukin 12 p40; NLRP3 = NLR family pyrin containing 3; Tf = transferrin, TGFβ = Transforming growth factor-beta; TNFα1 = Tumor necrosis factor-alpha-1; TNFα2 = Tumor necrosis factor-alpha-2; SAA = Serum Amyloid A

CHAPTER 4

GENE EXPRESSION ANALYSIS OF THE ACUTE PHASE RESPONSE IN GOLDFISH (*Carassius auratus* L.) INFECTED WITH *Trypanosoma carassii*[1]

4.0 Introduction

An integral component of the innate immunity is the acute phase response (APR), which is a rapid physiological reaction to infection, injury or trauma [2]. The pathogen-induced homeostatic disruption leads to the release of cytokines and inflammatory mediators such as IL-1, IL-6 and TNF-α, which induce rapid synthesis of acute phase proteins (APP) in the liver [3,81]. A significant change in the blood plasma composition of acute phase proteins (APPs) characterizes the APR, enabling the host to limit pathogen growth and restore homeostasis.

APPs have various functions such as the ability to opsonize pathogens and activate the complement system, scavenge free radicals, protect against oxidative damage and neutralize enzymes [3]. APPs are classified according to a variety of parameters including: magnitude of concentration change in plasma (major, intermediate, minor), direction of concentration change (negative versus positive) and function (i.e. transport, interaction with complement components etc.) [2]. Serum amyloid A (SAA) and C-reactive protein (CRP) are major APPs whose concentrations increase as much as 1000-fold during inflammation [125]. CRP has been shown to enhance phagocytosis of pathogens and activate classical complement pathway [97]. The functions of SAA are not well established, however, SAA has been implicated in chemotactic recruitment of inflammatory cells, promotion of the expression and eventual production of pro-inflammatory cytokines and the regulation of adaptive immune responses [89,124].

[1]A portion of this chapter has been published: Kovacevic, N., Hagen, M.O., Xie, J., and Belosevic, M. 2015. The analysis of the acute phase response during the course of *Trypanosoma carassii* infection in the goldfish (*Carassius auratus* L.). *Developmental and Comparative Immunology* **53(1):** 112-122

Ceruloplasmin (Cp), hemopexin (Hx), and transferrin (Tf) are classified as transport APPs, involved in the regulation of iron concentrations in the host [352].

In human and veterinary medicine, APPs are well characterized and changes in their concentrations in the host are handy diagnostic tools for identifying ongoing infection and inflammation, as well as evaluation tools for assessment of immunotoxicity [4,5,361].

Research of host defense in fish has attracted widespread attention due to a growing aquaculture industry, as disease outbreaks from overcrowding and associated stress cause devastating losses [6]. Amongst bacterial and viral infections, parasites pose a serious risk to aquaculture. Adapting parasites to laboratory models is difficult, limiting the knowledge of innate immune responses, and in particular the APR during infection in teleosts [8]. Our laboratory has a natural host-parasite model system that is be used to assess numerous parameters of this relationship. *Trypanosoma carassii* is a protozoan, extracellular parasite infecting economically important cyprinids [10]. In aquaculture setting, prevalence of infection can reach up to 100%, resulting in significant morbidity and mortality [281].

In this study, I examined the expression of genes that encode APPs to assess the importance of these during the course of *T. carassii* infection in the goldfish.

4.1. Experimental design

4.1.1. Course of infection in goldfish infected with *T. carassii*

Individual fish (*n*=6 for each group) were anaesthetized with TMS and injected intraperitoneally with 6.25×10^6 *in vitro* grown parasites suspended in 100 µL of TDL-15 medium, or sham injected with 100 µL of TDL-15 (control fish). Fifty microliters of blood were collected on days 3, 7, 14, 21, 28 and 56 after infection, and 4 µL of undiluted blood was added to 96 µL of tri-sodium citrate anticoagulant (100 mM tri-sodium citrate, 40 mM glucose, pH 7.3). Parasitemia was enumerated using the methods described previously [281,355]. Briefly, parasitemia was enumerated as parasites/mL of blood using a haemocytometer. When parasite numbers were too low to detect using a haemocytometer, hemocrit centrifugation technique was used [356]. Number of parasites

per mL was log transformed and presented as mean ± SEM parasites per mL of blood of (n=6) fish per experimental group.

4.1.2 Identification of goldfish acute phase proteins

CRP primers were designed based on the nucleotide sequences against the CRP gene of common carp (Accession No. AB028455.1) and zebrafish (Accession No. JF772178.1). RACE PCR (Clonetech, USA) was done to obtain the open reading frame and untranslated 5' and 3' sequences of goldfish CRP. To determine the sequence CRP, it was first cloned into pJET1.2/blunt cloning vector (Fermantas) and colonies containing the correct insert were identified by colony PCR using pJET1.2 sequencing primers (Fermantas). Positive clones were isolated using the QIAprep spin Miniprepkit (Qiagen) and sequenced using the DYEnamic ET terminator cycle sequencing kit and a PE Applied Biosystems 377 automated sequencer. Sequences were analyzed using 4peaks software and BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences of goldfish CRP (Accession No. KP057691) has been submitted to the Genbank.

Goldfish Tf and Hx (syn. Wap65) have been previously cloned and sequenced [165,192], and Cp (Accession No. HM355584) has been partially sequenced. Quantitative PCR primers were designed using Primer Express Software (Applied Biosystems). Primers were validated by assessing the relative quantitative efficiency against a curve designed from serial dilutions of goldfish cDNA. Primers were chosen if the R^2 values were greater than 0.980 and an efficiency > 90%. To confirm primer specificity, qPCR products were run on a gel and sequenced.

4.1.3 Assessment of the effects of *T. carassii* infection on expression of acute phase proteins of goldfish

Kidney, liver and spleen were removed from non-infected control and infected (n=6) goldfish on 3, 7, 14, 21, 28 and 56 days post infection. Samples were immediately flash frozen in liquid nitrogen and stored at -80°C until isolation of RNA.

Kidney, spleen, and liver RNA was extracted from tissues using TRIzol reagent (Invitrogen) or RNAeasy Mini Kit according to manufacturer's instructions. The purity of

the RNA was confirmed by assessing the ratio of the absorbance at 260 nm and 280 nm as well as 260 nm and 230 nm. 2.5 µg of total RNA was reverse transcribed into cDNA using Superscript III cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. The cycling parameters were as follows: 95ºC, 2 minutes; 95ºC, 15 seconds; 60ºC, 1 minute; 95ºC, 15 seconds; 60ºC, 1 minute; 95ºC, 30 seconds; 60ºC, 15 seconds (melting curve); for 40 cycles.

The fold difference (RQ) was determined as described by the qPCR instruction manual (Applied Biosystems); the endogenous control gene, was elongation factor 1 alpha (EF-1α). All expression data were normalized using the baseline expression in non-infected control fish for each gene. All samples were run in triplicate and presented as the mean ± SEM of 6 fish per experimental group.

4.2 Results

4.2.2 The course of *T. carassii* infection in goldfish

All fish injected with the parasites became infected as indicated by examination of their blood for trypanosomes as early as 3 days post infection (dpi) (Table 4.1). Parasite numbers peaked at 14 dpi with a mean log parasite burden of 6.16 ± 0.35 parasites/mL. Thereafter, parasite numbers begins to progressively decline with a mean log burden of 3.97 ± 0.58/mL by 56 dpi. The first phase of infection (up to 21 dpi) is referred to as the acute phase, after which the following phase is referred to as the chronic or elimination phase (days 28-56). No mortality was observed in both infected and non-infected fish.

4.2.3 *T. carassii* alters the expression of acute phase proteins during the acute phase of infection in goldfish

To understand the role acute phase proteins have during *T. carassii* infection of goldfish, I measured the expression of several acute phase proteins: SAA, CRP, Hx, Tf and Cp using quantitative RT-PCR and compared expression in the kidney, liver and spleen in infected and non-infected goldfish.

In the liver, infected fish had increased mRNA levels of CRP, SAA and Tf during the early course of infection, when compared to control fish (Fig. 4.1A-D). The mRNA

levels of Cp, CRP, SAA and Tf were highest at 7 dpi (Fig. 4.1B), and Cp and Tf mRNA levels remained statistically significant compared to control non infected fish at 21 dpi (Fig. 4.1D), whereas SAA mRNA levels in the liver were similar to those in controls at 21 dpi. By 28 and 56 dpi, the expressions of all APPs were similar to those in non-infected age-matched non-infected fish (Fig. 4.1E-F), with the exception of SAA that was down-regulated at 28 dpi (Fig. 4.1E).

In the kidney, CRP, SAA and Tf mRNA levels were significantly up-regulated by 3 dpi (Fig. 4.2A). CRP and Tf expression retuned to control levels by 14 dpi (Fig. 4.2B-C), whereas CRP mRNA levels were significantly different in infected fish on 21 dpi and were 4-fold higher than those in and age-matched non-infected control fish (Fig 4.2D). The SAA mRNA levels were significantly elevated on days 3, 7 and 14 dpi and were similar to those in non-infected fish on 21 dpi (Fig. 4.2C-D). At 56 dpi, SAA and Tf mRNA levels were up-regulated compared to those in control fish (Fig. 4.2F).

In the spleen, SAA mRNA levels were 20-fold higher in infected compared to non-infected fish at 7 dpi (Fig 4.3A-D). In contrast, Cp, Hx, and Tf were significantly down-regulated at 7 dpi in the spleen and returned to control levels by 21 dpi. The CRP mRNA levels did not change in the spleen until 21 dpi (Fig. 4.3D). At day 28, the expressions of genes encoding APPs were comparable to those in control fish (Fig. 4.3E), whereas by 56 dpi, there was a 10-fold higher gene expression of Cp, Hx, and Tf compared those in control fish (Fig. 4.3F).

4.3 Discussion

The course of infection reported in my thesis is consistent with previously published reports on the course of infection with *T. carassii* [12,257,288]. The *in vitro* cultured parasites were infective since all inoculated fish developed trypanosome infections as early as 3 dpi. No mortality was observed, and this has been attributed to loss of virulence in prolonged *in vitro* cultures of *T. carassii.*

In this study, I used an *in vivo* goldfish model system to assess APP gene expression during the course of infection of goldfish with *T. carassii.* The gene expression analysis indicated that *T. carassii* induced significant changes in APP gene

expression in the liver, kidney, and spleen during the infection. Similar up-regulation in the expression of genes encoding APPs has been observed in other teleosts infected with different pathogens. In channel catfish infected with the bacterium *Edwardsiella ictaluri*, APPs were significantly up-regulated in the liver [6]. Specifically, APPs involved in iron metabolism such as Cp and Tf were up-regulated as early as 3 dpi, which is similar to what I observed in our study. In zebrafish infected with *Aeromonas salmonicida* and *Staphylococcus aureus* increased gene expression of a CRP-like molecule was observed as early as one hour post infection and increased expression of SAA at 1 dpi [151].

In the host, iron levels are tightly regulated to ensure a proper balance between adequate nutrition and toxicity. Iron is not only an essential nutrient for the host, but it is also necessary for proper growth and development of pathogens [327]. Thus, regulation of iron availability is an important host defense strategy. Ceruloplasmin (Cp), hemopexin (Hx), and transferrin (Tf) are classified as transport APPs and participate in the regulation of iron levels in the host [2,352]. Ceruloplasmin, a serum ferroxidase, oxidizes ferrous iron (Fe^{2+}) to the useable ferric iron (Fe^{3+}) [169]. Transferrin is a glycoprotein, which carries ferric iron (Fe^{3+}). This complex is then bound by transferrin receptors on target cells, allowing iron uptake [158]. Hemopexin, or more commonly known as its homologue Warm-acclimation protein 65 (WAP65), binds excess heme-Fe^{2+} for transport to the liver, where it is recycled [362].

My findings on the gene expression analysis suggest iron-withholding strategies may be utilized as part of the APR due to up-regulation of iron regulatory proteins seen in the liver. Similar results were obtained in a study assessing transcript levels in channel catfish infected with *E. ictaluri* where APPs were up-regulated in the liver [6]. Rainbow trout infected with a *Vibrio* sp. showed increased transcripts of iron regulatory molecules, hepcidin and haptoglobin [187]. Increased Tf can limit pathogen growth and survival by creating environments with decreased iron availability [363]. In our study, Cp and Tf were significantly up-regulated from 3 dpi until 21 dpi in the liver. In contrast, carp infected with *I. multifiliis* did not induce changes in Tf mRNA expression in skin, liver or blood [342]. In sea bass infection with *Photobacterium damselae*, there was decreased Tf expression in the liver [164]. This is similar to mammals, where Tf was generally down-regulated in response to infection, and it differs from reports that showed that in avian

species Tf expression was up-regulated in the liver [364]. Thus, the differences in Tf gene expression in the liver upon pathogen challenge may be dependent on both host and pathogen. During the later stages of the infection of goldfish with *T. carassii*, the expression of Cp and Tf returned to levels similar to those in control fish. These observations are similar to what has been reported for *Trypanosoma brucei* infected mice, where gene expression Cp and Tf returned to baseline during later stages of the infection [333]. Typically, the APR is induced until homeostasis is restored or is in the process of being restored. In the *T. carassii* infected goldfish, parasite numbers begin to decrease by 21 dpi, and compared to the parasitemia at 14 dpi, there was up to a 3 log reduction in the number of parasites by 56 dpi, which parallels the down-regulation in APP gene expression in the liver.

A persistent up-regulation of SAA was observed throughout the acute course of *T. carassii* infections in all tissue examined. SAA and CRP have important immune roles and have been implicated in linking the innate and the adaptive immune systems. SAA acts as a cytokine-like immunomodulator [128] to induce cytokine production, making it a potent pro-inflammatory mediator [365]. Despite the plethora of research on SAA and its various effects, its physiological functions are yet to be fully elucidated. To date, no SAA-deficient animals have been generated, suggesting that it may play a vital role in the survival of multicellular organisms [124]. Our results support earlier findings of the increases SAA gene expression in the liver and kidney of common carp infected with the *Trypanoplasma borreli* [153], and Asian sea bass infected with *Cryptocaryon irritans* [152].

In goldfish spleen, SAA was highly expressed throughout the course of infection. This is similar to findings reported for Atlantic salmon infected with *A. salmonicida*, where the SAA expression was significantly elevated on 13 dpi [155]. In contrast, SAA was down-regulated on 10 dpi in the spleen of Asian sea bass infected with *C. irritans* [152]. There are also reports that SAA expression was highly variable upon parasite challenge [154], suggesting that changes in SAA expression in host tissues may be pathogen-dependent.

In this study, CRP was up-regulated during the acute part of infection (3 and 7 dpi) in the liver and kidney, and at 21 dpi in the spleen. In mammals, CRP is primarily

synthesized in the liver during the APR [97]. However, there are several reports of circulating CRP deposits at sites of inflammation or tissue damage [366]. *T. carassii* has been reported to cause substantial changes in hematopoietic organs such as disintegration of red blood cells in both the kidney and spleen. In addition, *T. carassii* aggregates and is sequestered in internal organs such as the kidney and contributes to a continual priming of the immune system [279,287,288], which may contribute to changes in CRP expression in these tissues.

Similar up-regulation of CRP has been observed in other fish species infected with pathogens or exposed to chemicals. For example, in Atlantic cod (*Gadus morhua* L.) stimulated with turpentine oil a significant up-regulation of CRP was observed in the kidney [367]. An increase in CRP expression was also observed in zebrafish exposed to *A. salmonicida* and *S. aureus* [151], and rainbow trout exposed to *Yersinia ruckeri* O1 [368]. In common carp, infected with *A. hydrophila,* there was an increase in serum CRP [369], and rohu and major carp exposed to various metals had higher CRP-like protein levels in the serum [370].

CRP has been reported to recognize PAMPs such phosophorylcholine that is present on the surface of parasites. Binding of CRP to these parasites moieties apparently confers anti-parasitic properties, however the mechanism(s) are unclear [9,371]. In addition to its anti-parasitic properties, CRP acts as a pro-coagulant that may hinder the ability of the extracellular parasite to establish and proliferate in the host [371,372].

APPs are important component of early response to infectious agents not only in mammals but also teleosts. The diverse functional properties of APPs ensure a more efficient control of infectious diseases, and as such constitute an essential role in innate host defense against prokaryotic and eukaryotic pathogens.

Table 4.1 Parasitemia in goldfish infected with *Trypanosoma carassii*[1]

Days Post-Infection	Number of Trypanosomes/mL (log_{10}) (Mean ± SEM)
3	4.91 ± 0.48
7	5.25 ± 0.43
14	6.16 ± 0.35
21	5.92 ± 0.25
28	5.10 ± 0.31
42	4.30 ± 0.57
56	3.97 ± 0.58

[1]Fish (*n*=6) were inoculated with 6.25 X 10^6 of *in vitro* grown *T. carassii.* Blood samples were collected for individual fish throughout the course of infection and the parasite load was enumerated. The results are mean of parasites per mL of blood ± SEM.

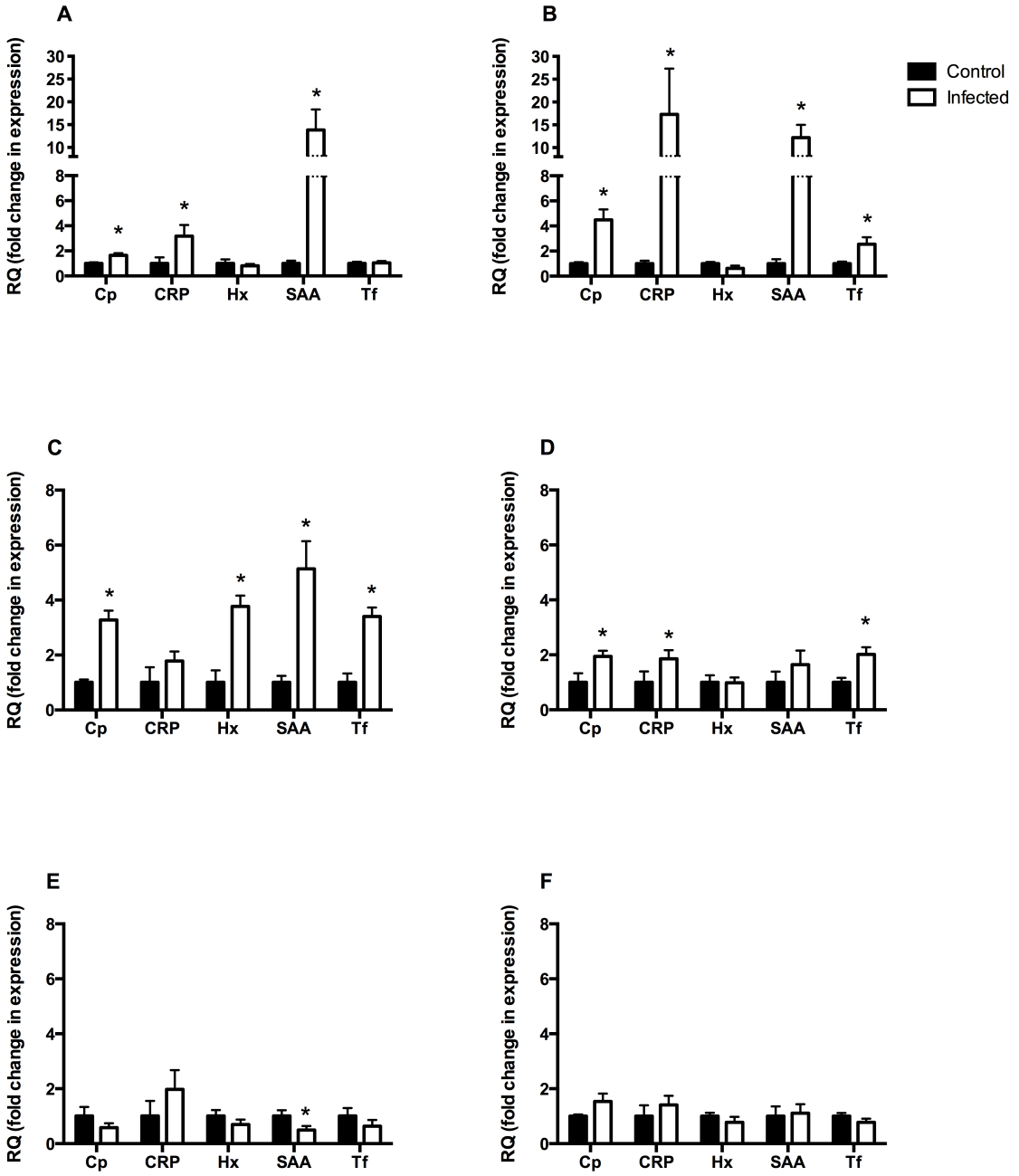

Figure 4.1 Quantitative expression analysis of acute phase protein encoding genes in the liver of *T. carassii* infected and non-infected goldfish: (A) 3 days post infection (B) 7 days post infection (C) 14 days post infection (D) 21 days post infection (E) 28 days post infection and (F) 56 days post infection. Expression was relative to EF-1α and normalized against those observed in non-infected control fish for each gene. Data are represented as mean ± SEM of 6 fish ($P < 0.05$). Asterisks (*) denote statistical significance from non-infected control fish. (CRP = C-reactive Protein; SAA = Serum Amyloid A; Tf = Transferrin).

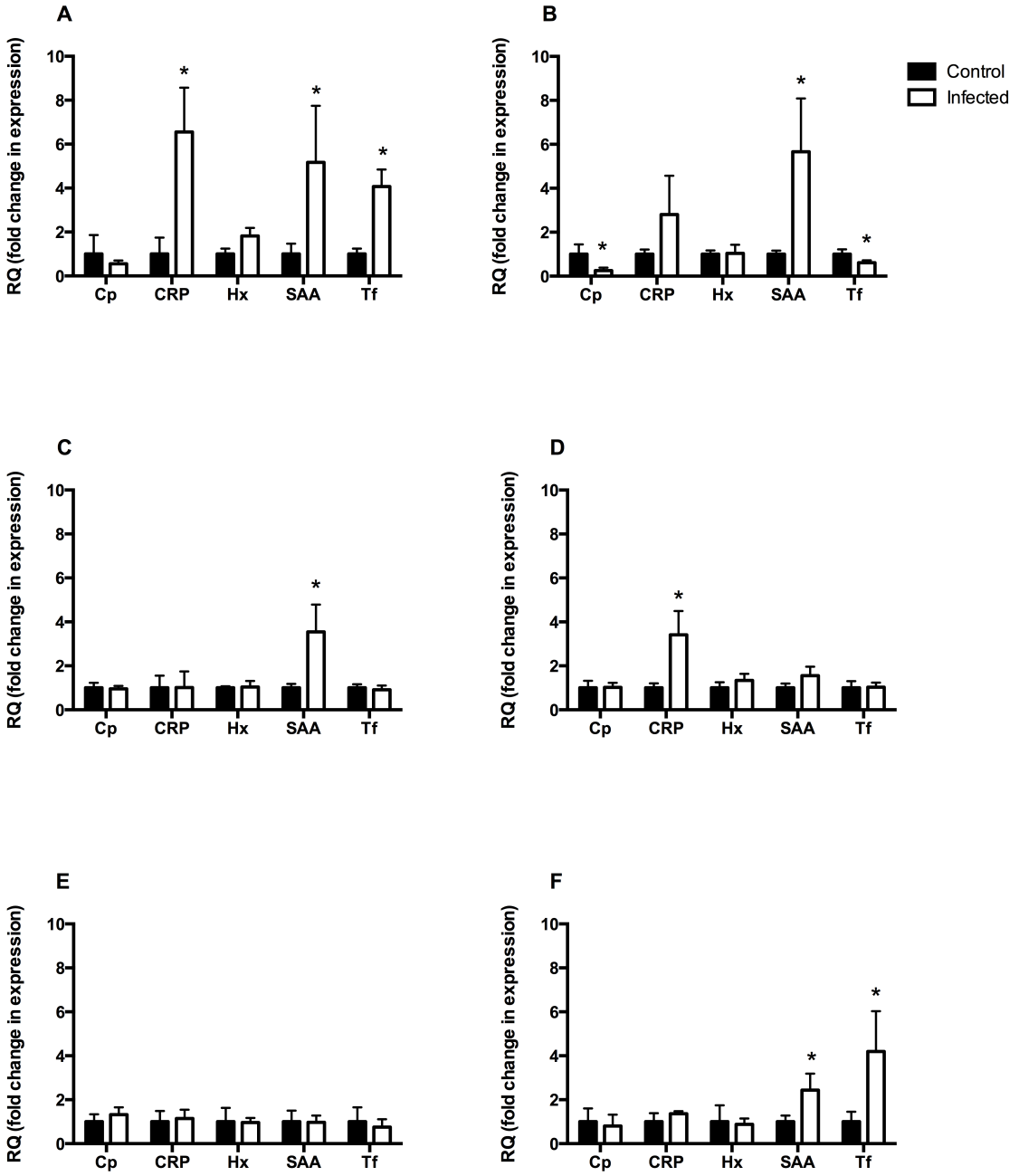

Figure 4.2 Quantitative expression analysis of acute phase protein encoding genes in the kidney of *T. carassii* infected and non-infected goldfish (A) 3 days post infection (B) 7 days post infection (C) 14 days post infection (D) 21 days post infection (E) 28 days post infection and (F) 56 days post infection. Expression was relative to EF-1α and normalized against those observed in non-infected control fish for each gene. Data are represented as mean ± SEM of 6 fish ($P < 0.05$). Asterisks (*) denote statistical significance from non-infected control fish. (Cp = Ceruloplasmin; CRP = C-reactive Protein; Hx = Hemopexin; SAA = Serum Amyloid A; Tf = Transferrin)

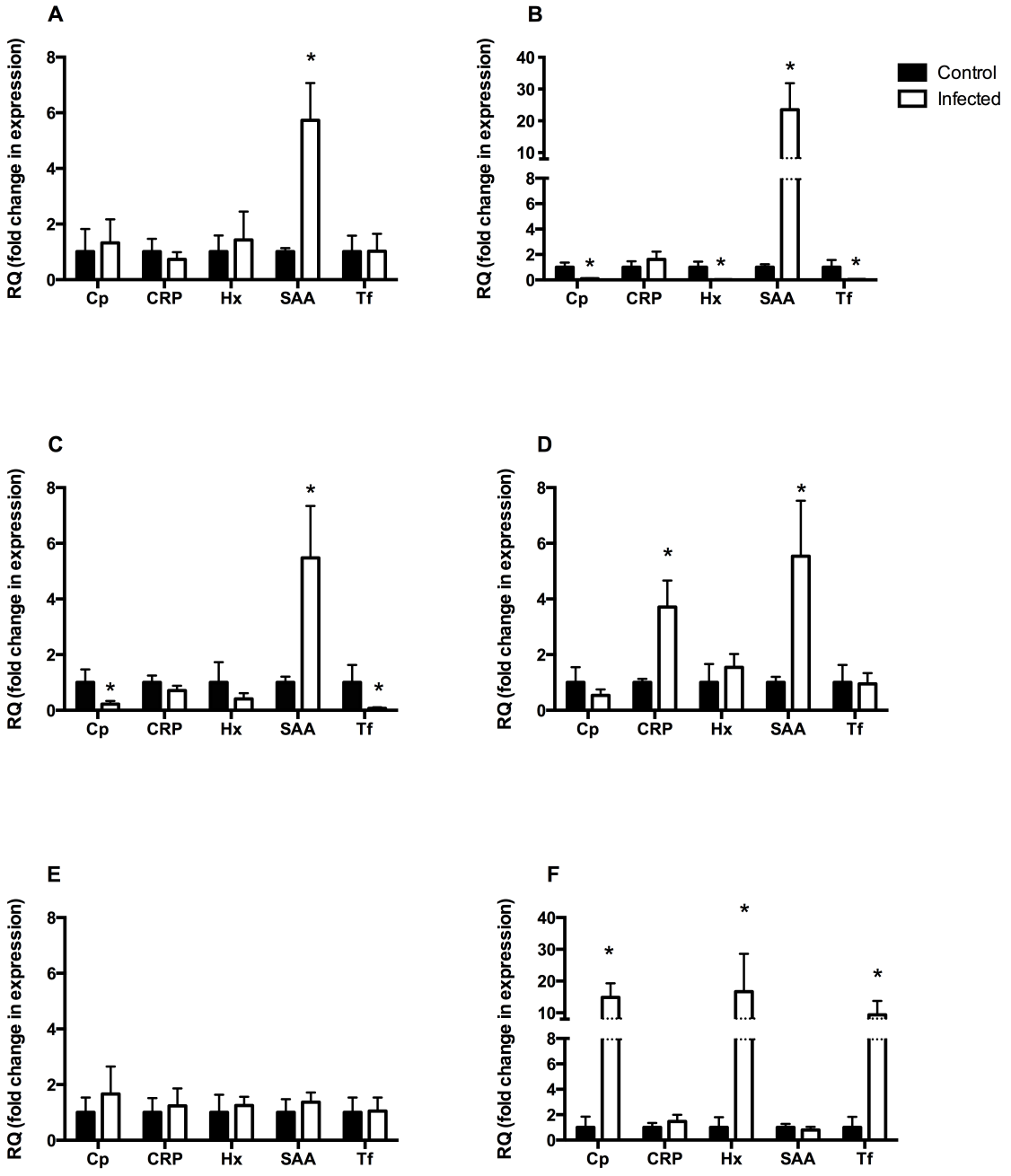

Figure 4.3 Quantitative expression analysis of acute phase protein encoding genes in the spleen of *T. carassii* infected and non-infected goldfish: (A) 3 days post infection (B) 7 days post infection (C) 14 days post infection (D) 21 days post infection (E) 28 days post infection and (F) 56 days post infection. Expression was relative to EF-1α and normalized against those observed in non-infected control fish for each gene. Data are represented as mean ± SEM of 6 fish ($P< 0.05$). Asterisks (*) denote statistical significance from non-infected control fish. (Cp = Ceruloplasmin; CRP = C-reactive Protein; Hx = Hemopexin; SAA = Serum Amyloid A; Tf = Transferrin)

CHAPTER 5

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF GOLDFISH C-REACTIVE PROTEIN[1]

5.0 Introduction

C-reactive protein (CRP) is a classical serum pentraxin [361] composed of 5 subunits arranged in a pentagonal shape [103]. It was characterized by calcium-dependent binding to ligands and high affinity to the phosphocholine (PC) moiety of C-polysaccharide [105,118]. During an inflammatory episode or insult, CRP concentrations can increase significantly making it a reliable biomarker of health status in humans and mammalian species.

CRP has been proposed as having numerous immunological roles. Human CRP can bind damaged and apoptotic cells via phospholipids such as PC to promote removal in an anti-inflammatory manner [373]. In addition to binding the PC moiety of C-polysaccharide, CRP has been reported to bind microorganisms such as fungi, bacteria, and intracellular parasites (*Plasmodium, Leishmania)* [95,374]. This interaction allows CRP to act as an opsonin enhancing phagocytosis and clearance of microbes [110]. Complement activation may be a mechanism by which apoptotic cells and microbes are removed [375]. CRP can activate the classical complement cascade by binding to component C1q [9]. Furthermore, CRP has been documented to bind noxious compounds such as mercury to detoxify it [376]. CRP shows high conservation in structure and binding abilities, making it a primitive molecule of innate immune protection [82]. The various functions of CRP make it an especially important aspect of innate defense in lower vertebrates such as teleosts [377].

The presence of CRP has been reported in numerous teleost species, including rohu (*Labeo rohita)* [370], plaice (*Pleuronectes platessa)* [378], rainbow trout (*Oncorhynchus*

[1]A portion of this chapter has been published: Kovacevic, N., Hagen, M.O., Xie, J., and Belosevic, M. 2015. The analysis of the acute phase response during the course of *Trypanosoma carassii* infection in the goldfish (*Carassius auratus* L.). *Developmental and Comparative Immunology* **53(1):** 112-122

mykiss) [379], common carp *(Cyprinus carpio)* [380], and zebrafish (*Danio rerio*) [381]. In many of these studies, researchers have focused on identification and expression of CRP in fish species. CRP has anti-parasitic properties [9], however not many studies have assessed the CRP response to parasitic infections.

In this chapter, I report on the identification and functional characterization of goldfish CRP against *T. carassii.* Goldfish CRP was highly expressed in spleen, with lower mRNA levels observed in other tissues examined. In non-stimulated goldfish immune populations, mRNA levels of CRP were highest in monocytes, splenocytes and neutrophils, with lowest expression observed in macrophages. A recombinant form of goldfish CRP (rgfCRP) was produced using a prokaryotic expression system. Treatment of goldfish monocytes and macrophages did not induce antimicrobial responses against *T. carassii.* However, addition of rgfCRP enhanced complement-mediated killing of trypanosomes *in vitro*, and the lysis increased after addition of immune serum.

5.1 Experimental design

5.1.1 Molecular cloning and expression of rgfCRP

The sequence for goldfish *CRP* was identified as previously described [382]. Briefly, primers were designed based on the nucleotide sequences against gene of common carp (Accession No. AB028455.1) and zebrafish (Accession No. JF772178.1). RACE PCR (Clonetech, USA) was done to obtain the open reading frame and untranslated 5' and 3' sequences of goldfish CRP.

PCR fragment encoding the full sequence of goldfish CRP ORF was amplified using primers that allow cloning into pET SUMO expression vector (Invitrogen). PCR products were gel purified (QIAquick Gel Extraction Kit, Qiagen) and ligated into pET SUMO vector (Invitrogen). The recombinant plasmid was transformed into competent *Escherichia coli* (NEB10, Biolabs) and plated onto LB-kanamycin plates (50 μg/mL) and incubated at 37°C overnight. Positive clones were identified by colony PCR, cultured in LB-kanamycin, and plasmid DNA purified from recombinant clones using QIAprep spin Miniprepkit (Qiagen). To verify that the insert was in the correction orientation and frame, the purified plasmids were sequenced using vector specific primers. The pET SUMO

vector encodes an N-terminal 6XHis tag for purification and detection of the recombinant molecules.

5.1.2 Quantitative expression of CRP in goldfish normal goldfish tissues and non-stimulated immune cells

Quantitative gene expression of CRP was assessed in normal goldfish tissues (kidney, spleen, liver, intestine, brain, muscle and gill) as well as goldfish immune cell populations (mature macrophages, monocytes, neutrophils, splenocytes, and PBLs). Total RNA was isolated using TRIzol Reagant (Invitrogen) and reverse transcribed into cDNA using SuperScript III cDNA kit (Invitrogen) according to the instructions by the manufacturer. The RQ values were normalized against the lowest expression of APPs observed in goldfish tissues (muscle) and immune cell populations (macrophages). The cycling conditions were the same as those described in Section 3.5.2

5.1.3 Infection of fish with trypanosomes

Fish were anaesthetized with TMS and were injected intraperitoneally 6.25 x 10^6 or 1×10^7 *in vitro* grown parasites suspended in 100 µL of TDL-15 medium, or sham injected with 100 µL of with TDL-15 (control fish). Fifty microliters of blood was removed on days 3, 7, 14, and 21, and 4 µL of undiluted blood was added to 96 µL of tri-sodium citrate anticoagulant (100 mM tri-sodium citrate, 40 mM glucose, pH 7.3). Parasitemia was enumerated using the methods described previously [281,355]. Number of parasites per mL was log transformed and represented as mean ± SEM $\log_{10}$ parasites per mL of blood of (n=6) fish per experimental group.

5.1.4 Quantitative expression of CRP in non-infected, low *T. carassii* infected and high *T. carassii* infected goldfish during acute infection

Kidney, liver and spleen were removed from non-infected control (n=6) and both doses infected (n=6 each) goldfish on 3, 7, 14 and 21 dpi. Samples were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA processing.

Kidney, spleen, and liver RNA was extracted from tissues using TRIzol reagent (Invitrogen) or RNAeasy Mini Kit according to manufacturer's instructions. 2.5 µg of total RNA was reverse transcribed into cDNA using Superscript III cDNA synthesis kit

(Invitrogen) according to manufacturer's instructions. The cycling conditions are the same as described in Section 4.1.3. The fold difference (RQ) was found as instructed by the qPCR instruction manual (Applied Biosystems) in comparison to the endogenous control gene, elongation factor 1 alpha (EF-1α). All samples were run in triplicate and presented as the mean ± SEM of 6 fish per experimental group.

5.1.5 Scale up production of recombinant goldfish CRP (rgfCRP)

Plasmids containing in frame insert of CRP was transformed into BL21 (DE3) One Shot *E. coli* cells (Invitrogen), scaled up to mid-log phase (OD_{600} of 0.5-0.6), and induced with 1 mM IPTG for 3 hours. Optimal induction time and concentration was determined in a pilot study. The induced bacteria was pelleted and frozen at -20°C until needed. The bacteria resuspended in lysis buffer (2.5 mL of 10X FastBreak Lysis Reagant (Promega) in 22.5 denaturing wash buffer (100 mM Hepes, 10 mM imidazole, 7.5 M urea, pH 7.5), and incubated with MagneHis Ni-particles (Promega) as recommended by manufacturer. A PolyATtrack System 1000 magnet (Promega) was used to retain Ni-particles bound to recombinant gfCRP. Protease Inhibitor cocktail (500 μM AEBSF, HCl, 150 nM Aprotonin, 1 μM E-64, and 1 μM Leupeptin) was added to all buffers to prevent protein degradation. The supernatants were discarded, the beads washed 3 times under denaturing conditions (as described above), and the recombinant proteins eluted from the beads using 500 mM imidazole. To confirm the presence of recombinants, a Western blot was performed using the anti-6X His tag antibody.

The recombinant protein was renatured overnight in 10 volumes of re-naturation buffer (4 mM reduced glutathione, 2 mM oxidized glutathione, 50 mM sodium borate, 5 mM EDTA, pH 8.5), and dialyzed overnight against 4 L of 1X PBS overnight using 20 kDA MWCO Snakeskin dialysis tubing (Pierce). To concentrate protein, the dialysis tubing was placed in polyethylene glycol flakes for 8 hours and further dialyzed against PBS overnight to remove traces of imidazole and urea). The N-terminal His- and SUMO-tag were cleaved from recombinant CRP using SUMO protease (Invitrogen) and purified using MagneHis Ni-particles. The endotoxin was removed from the recombinant protein using Pierce High Capacity Endotoxin Columns (Thermo Scientific) according to

manufacturer's directions. Recombinant protein was analyzed using the *Limulus* amebocyte lysate kit (Thermo Scientific) and found to be endotoxin free. Protein concentration was determined using a Micro BCA Protein Assay Kit (Thermo Scientific).

5.1.6 *In vitro* binding assay

Recombinant CRP (2.5 μg) was suspended in conjugation buffer (20 mM Hepes) containing either PBS or varying concentrations of calcium chloride (1.75 mM, 2.5 mM or 5 mM) for 15 minutes. rgfCRP was cross-linked using di-succinimidyl suberate (DSS, Thermo Scientific) according to manufacturer. Cross-link reaction was allowed to proceed for 30 minutes, after which it was terminated by addition of 50 mM Tris. The reactions were resolved using reducing SDS-PAGE and western blot using polyclonal rgfCRP antibody and visualized using ECL detection Kit (Pierce).

5.1.7 Assessment of rgfCRP on nitric oxide response of goldfish macrophages

Nitric oxide (NO) assay was performed as performed as previously [383]. Briefly, macrophages from 6 to 8 day cultures were established using cells from 3 individual fish and seeded in wells of 96-well plates at a density 2 x 10^5 cells/well. These were then incubated in culture medium alone (control) or treated with one of the following: heat killed *A. salmonicida*, 1 μg/mL or 5 μg/mL of cleaved and un-cleaved rgfCRP, with or without 1 x 10^6 *T. carassii*, and incubated at 20°C for 48 hours. Nitrite production was determined using the Griess reaction. Briefly, 1% sulphanilamide and 0.1% N-napthyl-ethylenedamine were added to cultures and nitrite levels were determined colorimetrically at 540 nm and a nitrite standard curve.

5.1.8 Assessment of rgfCRP on respiratory burst response of goldfish monocytes

Respiratory burst assay was performed as previously described [383]. Briefly, goldfish monocytes from 3 to 4 day old separate cultures of cells obtained from 3 individual fish were seeded into 96-well plates at a density of 2 x 10^5 cells/well and incubated in culture medium alone (control) or treated with one of the following: heat killed *Aeromonas salmonicida*, 1 μg/mL or 5 μg/mL of cleaved and un-cleaved rgfCRP, with or without 1 x 10^6 *T. carassii*, and incubated for 16 hours at 20°C after which NBT (2 mg/mL, Sigma) and PMA (final triggering concentration 100 ng/mL; Sigma) in PBS

were added to the cultures and incubated at room temperature for 30 minutes. The plates were centrifuged (400 x *g*, 10 minutes), supernatants aspirated, and pelleted cells were fixed using methanol. Non-reduced NBT was removed by washing twice with methanol and reduced NBT was dissolved by 2 M KOH. To induce a colorimetric response, DMSO was added and plates read at 630 nm. To factor in background NBT reduction, absorbance from cells alone (no PMA) was subtracted from treatment group values.

5.1.9 Assessment of rgfCRP to enhance phagocytosis of *T. carassii* in macrophages

5.1.9.1 Preparation of *T. carassii* and opsonisation of targets with rgfCRP

T. carassii was labelled with 500 ng/mL FITC with continuous shaking at room temperature for 30 minutes. After staining, *T. carassii* was washed twice with 1×PBS *E.coli* DH5α-GFP was washed twice with 1X PBS before use in experiments. *T. carassii*-FITC and *E.coli* DH5α-GFP were opsonized with either: 0.5 and 5 µg/mL rgfCRP or a PBS control, after which targets were washed twice with 1X PBS to remove unbound particles.

5.1.9.2 Phagocytosis Assay using ImageStream

E.coli DH5α-GFP, T.*carassii*-FITC or rgfCRP opsonized *T.carassii*-FITC or *E.coli* DH5α-GFP were added to 1 x 10^5 macrophages at a ratio of 5:1 (bacteria/parasite: cells), and incubated for 2 hours at 20°C. After incubation, the samples were fixed in 1% formaldehyde at 4°C for 20 minutes. Data were collected using the ImageStream multi-spectral imaging flow cytometer (Amnis) and analyzed using INSPIRE software. A minimum of 10,000 cells was acquired. Phagocytic index was calculated based on the number of particles/parasites internalized per macrophage.

5.1.10 Assessment of rgfCRP to enhance complement lysis of *T. carassii*

5.1.10.1 Enzyme treatment of trypanosomes

Trypanosomes cultured *in vitro* were washed twice in serum-free medium and re-suspended to 1 x 10^6/mL. Medium alone or 0.1 mg/mL trypsin (Trypsin (TPCK-treated), Sigma) treatments were added to 1 mL of trypanosome suspension and incubated for 1 hour at 20°C. Subsequently, 10% HI-GFS or 0.1 mg/mL Soybean trypsin inhibitior:SBTI

(Sigma) was added to the reaction tubes and the tubes incubated for 10 minutes at 20°C. After enzyme inactivation, trypanosomes were washed twice in serum-free medium and re-suspended in 1 mL of medium for use in the assay.

5.1.10.2 Trypanosome lysis assay

The procedure to assess trypanosomes susceptibility to lysis was described previously [313]. Briefly, 1 x 10^5 parasites were seeded in a 96-well plate in either medium, non-immune, heat-inactivated (HI) non-immune or immune goldfish serum or normal goldfish serum supplemented with either PBS or 0.5 μg/mL or 5 μg/mL of rgfCRP. Plates were incubated for 1 hour at 20°C, after which the contents of each well were re-suspended in medium and viable trypanosomes enumerated using a hemocytometer. Trypanosomes were considered viable when flagellar movement was observed.

5.1.11 Antibody production

Purified rgfCRP was used to immunize rabbits for production of polyclonal antibodies. Rabbits were injected with 250 μg purified rgfCRP mixed with 750 μL of Freund's Complete Adjuvant (FCA). Thereafter, booster injections were performed every 4 weeks up to 12 weeks using the same quantity of rgfCRP mixed with Freund's incomplete adjuvant (FIA). All inoculations and blood collection were performed by technicians at the University of Alberta Biological Sciences Animal Services Unit. The polyclonal IgG antibodies were purified from rabbit serum using Melon Gel IgG Spin Purification Kit (Thermo Scientific) according to manufacturer's specifications. The specificity of anti-rgfCRP IgG was confirmed using Western blot.

5.2 Results

5.2.1 Goldfish CRP *in silico* analysis

The complete open reading frames (ORFs) and the untranslated regions (UTR) of the goldfish CRP cDNA transcripts were obtained. GfCRP consisted of 936 bp with an ORF encoding 687 aa (Fig. 5.1). Conserved domains predicted by Pfam showed CRP had

a pentraxin domain (29-224 aa) and a conconavalin A-like lectin/glucanase domain (23-224 aa). In addition, goldfish CRP contain the pentraxin family signature (116-123 aa) H-x-C-[ST]-W-x-[ST], where the cysteine is involved in disulfide bonds.

Protein alignment of goldfish CRP with other known vertebrae sequences indicated that goldfish CRP amino acid sequence was highly conserved among species. All CRP protein sequences had a pentraxin domain (Fig. 5.2)

The phylogenetic analysis of CRPs of fish and select higher vertebrates is shown in Fig 5.3. Goldfish CRP branched closely to the teleost groups suggesting they are highly conserved among fish.

5.2.2 Analysis of CRP gene expression in normal goldfish tissues and non-stimulated goldfish immune populations

Assessment of goldfish CRP gene expression in the tissues of normal goldfish revealed the highest mRNA levels in the spleen, intestine and gill, and lowest mRNA levels in the muscle (Fig 5.4A). The mRNA levels of CRP were measured in different immune cell populations of normal goldfish (Fig. 5.4B). The mRNA levels were the highest in monocytes and splenocytes and lowest in macrophages and PBLs (Fig. 5.4B).

5.2.3 Assessment of parasitemia in goldfish infected with two doses of *T. carassii*

All fish injected with trypanosomes became infected as indicated by the examination of blood as early as 3 dpi (Table 5.1). Parasitemia peaked at 14 dpi in low *T. carassii* infected goldfish with a mean log burden of $6.16 \pm 0.35 \times 10^6$ parasites/mL, and thereafter declined progressively. Conversely, in the high *T. carassii* infected goldfish parasitemia continues to increase up until the last time point assessed (21 dpi) where parasitemia is significantly higher than low *T. carassii* infected goldfish (Table 5.1)

5.2.4 Assessment of CRP gene expression in goldfish infected with two doses of *T. carassii*

In the kidney, CRP was significantly up-regulated by 3 dpi in low dose infected fish, whereas in high dose infected goldfish, CRP expression was significantly down-regulated compared to control counterparts. At 7 dpi, only the high dose infected goldfish displayed significantly up-regulation of CRP mRNA levels compared to control

counterparts. At 21 dpi, both infection doses had significant up-regulation of CRP mRNA levels, however there were no significant differences between groups (Fig. 5.5A).

A similar trend was observed in the liver, where CRP mRNA levels were up-regulated by 3 dpi in low infected goldfish, and down-regulated in high *T. carassii* infected fish, in comparison to control counterparts (Fig. 5.5B). At 21 dpi, CRP mRNA levels were up-regulated for both infection dose groups, however they were no statistically significant differences between groups (Fig. 5.5B).

Similar to the kidney and spleen, CRP mRNA levels were significantly up-regulated at 21 dpi in both infection groups compared to non-infected control counterparts, however no significant differences were observed between infection groups (Fig. 5.5C). CRP mRNA levels were significantly up-regulated at 7 dpi in the high *T. carassii* compared to control non-infected fish, whereas low *T. carassii* infected fish displayed similar mRNA as control counterparts (Fig. 5.5C).

5.2.5 Goldfish CRP does not show pentamerization

Mammalian CRP has been shown to be present in the pentamer form. Goldfish CRP possesses a cysteine, which is involved in forming disulfide bonds in mammals. Therefore, I wanted to assess whether goldfish can form a pentamer, and whether the addition of calcium was necessary for these interactions. rgfCRP did not show any pentamerization on its own or with the addition of varying calcium concentrations (Fig. 5.6C).

5.2.6 Detection of goldfish CRP in sera of *T. carassii* infected goldfish

The specificity of anti-rgfCRP IgG was confirmed using Western blot (Fig. 5.6A). Subsequently, I wanted to assess whether CRP can be detected in sera collected from *T. carassii* infected goldfish was assessed using anti-rgfCRP. CRP was observed in 3 of the 5 fish assessed, each with varying levels of CRP detected (Fig. 5.6B). The presence of bands larger than the expected size of CRP (~25 kDa) was observed. This may be due to either aggregation or degradation of pentameric CRP which has been documented in mammals [384,385]. Due to lack of loading control, I was unable to quantify CRP levels in goldfish.

5.2.7 Recombinant goldfish CRP does not induce respiratory burst of monocytes and nitric oxide response of macrophages

The mammalian literature suggests that CRP (at concentrations <10 μg/mL) activate antimicrobial responses of macrophages [386]. To access the ability of rgfCRP to activate antimicrobial response in goldfish macrophages and monocytes, I measured the nitrite production and respiratory burst, respectively. The cleaved (His-tag removed) rgfCRP (Fig. 5.7A) or non-cleaved rgfCRP (Fig. 5.84A), alone or with trypanosomes, did not induce respiratory burst response in goldfish monocytes. Macrophages stimulated with either cleaved rgfCRP (Fig. 5.7B) or non-cleaved rgfCRP (Fig. 5.8B), alone or with trypanosomes, did not exhibit significant nitrite production compared to medium controls.

5.2.8 Recombinant goldfish CRP enhancement of complement mediated lysis

Cultures of trypanosomes incubated with non-HI (heat-inactivated) serum and 5 μg/mL rgfCRP showed a small, but significant reduction in the number of parasites. A greater reduction of parasites in cultures was observed when immune serum was added to the cultures; with enhanced reduction in viable parasites after addition of either 0.5 μg/mL or 5 μg/mL rgfCRP (Table 5.2). The reduction in the number of viable parasites was not observed in medium only group, or when heat-inactivated serum was added to the cultures. Trypsin-treated trypanosomes were highly susceptible to lysis with or without the addition of rgfCRP in the presence of normal serum (Table 5.2).

5.2.9 Recombinant goldfish CRP does not act as an opsonin to enhance phagocytosis

Opsonization with rgfCRP did not enhance phagocytosis of *T. carassii*-FITC or *E.coli* DH5α-GFP at either treatment of rgfCRP. *E.coli* DH5α-GFP displayed similar phagocytic index with or without rgfCRP, whereas there was no phagocytosis with *T. carassii* (Table 5.3).

5.3 Discussion

In this chapter, I report on the cloning and molecular characterization of goldfish CRP, and a comprehensive functional analysis of the molecule against a parasitic infection. CRP is used as a biomarker for diagnosis of infection, risk assessment and

prognosis. Levels of CRP have been correlated to bacterial load in several human studies [387,388]. Similar results have been seen in humans infected with parasitic infections such as malaria where CRP is used as a marker of morbidity [389,390]. CRP is commonly used in veterinary medicine to determine progression of disease and effectiveness of treatment [4]. In our experiment, the high dose *T. carassii* infected goldfish exhibited consistently higher CRP expression than low *T. carassii* infected goldfish with the exception of 3 dpi where there was a significant reduction in CRP in all 3 tissues assessed. At 3 dpi there was not a significant difference in parasitemia (Table 5.1), however the difference in inoculum load could have contributed to this observation. In mice infected with *Trypanosoma cruzi*, the parasite inocula influenced the activation of the immune response as well as the disease progression [391]. The authors report a higher IL-6 production in their medium *T. cruzi* inoculum group compared to a high inoculum group. Production of APPs such as CRP are strongly mediated by the potent inducer IL-6 [392,393]. This suggests that *T. carassii* inocula potentially have a role in modulating the immune response, in particular, IL-6 that would affect CRP production.

To assess whether goldfish CRP has similar antimicrobial effects as observed in mammalian counterparts, I cloned and molecularly characterized goldfish CRP. The goldfish CRP shared highest homology with common carp, and had the hallmark features present in all pentraxins, a pentraxin (PTX) domain [394], reported to recognize PAMPs such phosophorylcholine (PC), which is present on parasites and may confer anti-parasitic properties [371].

Quantitative gene expression analysis of goldfish CRP in normal goldfish tissues indicated highest expression levels in the spleen followed by intestine and gill (Fig. 5.4A). This differential expression was also observed in several other fish reports [114,380]. Organs such as spleen, intestine and gill of fish contain numerous immune cells, which have been reported to express CRP. In mammals, immune cells such as peripheral blood cells and macrophages have been shown to express CRP [395,396]. However, in goldfish non-stimulated cell populations I observed the lowest expression in PBLs and macrophages and highest expression in monocytes and splenocytes (Fig. 5.4B). CRP is a complement component [397], and it has been proposed that extrahepatic production of

complement components occurs in teleosts. This occurs in circulating blood, and lymph tissues awaiting potential pathogens as well as playing a role in clearance of apoptotic cells, and regeneration of cells and tissues [398].

CRP can activate the classical complement cascade by binding to component C1q [397]. I showed that in the presence of complement components of normal goldfish serum, parasite numbers are reduced *in vitro* (Table 5.2). This effect is abolished when serum is heat-inactivated suggesting heat-liable components of complement are necessary.

Previously, our lab showed that *T. carassii* was susceptible to the alternative complement pathway-mediated lysis [313]. My results indicate that the classical pathway of complement may also play a role in controlling parasite numbers *in vitro.* In common carp challenged with the virus CyHV-3, there was an up-regulation of complement related genes [123]. In addition, the authors found CRP serum levels to be correlated to complement activity in infected fish. However, this may not necessarily be the case *in vivo* where there are other factors at play. CRP can interact with complement regulatory proteins such as factor H. This interaction results in an inefficient generation of the membrane attack complex and abrogation of amplification of alternative pathway [97,399]. In addition, although CRP has anti-parasite properties, parasites have evolved mechanisms to evade host defenses. Parasites can bind CRP or C1q of the complement pathway to inhibit activation [9], allowing *T. carassii* to propagate and establish during early infection.

Phagocytosis of hemoflagellates has previously been reported in *Cryptobia salmositica* [400] and *Cryptobia (Trypanoplasma) bullock* [401]. However, phagocytosis of trypanosomes by macrophages has not been observed for *T. carassii* [12]. CRP has been shown to mediate phagocytosis of bacterial components such as *Vibrio angullarum* in tongue sole fish [114]. Phagocytosis of opsonized protozoan parasites such as *Leishmania donovani* [386] has been observed in mammals, however there is limited research in fish. In snapper, a pentraxin-like protein was able to opsonize and enhance phagocytosis of beads, however, this may be the result of passive adsorption instead of the active process of pattern recognition [122]. In this study, I observed no phagocytosis of *T. carassii* alone or with the addition of rgfCRP (Table 5.3). In addition, opsonisation

did not enhance phagocytosis of *E.coli* DH5α-GFP, suggesting there are differences in mammalian and fish CRP phagocytosis enhancement capabilities.

It has been reported that affinity-purified native human CRP induced the production of pro-inflammatory cytokines by cultured human monocytes [108]. Recombinant human C-reactive protein produced in *E. coli,* induced significant up-regulation of adhesion molecule expression in human endothelial cells, supporting the hypothesis that CRP may play a direct role in promoting the inflammatory component of atherosclerosis [402]. However, the induction of pro-inflammatory effects by CRP is controversial, since Verma and colleagues showed that human recombinant C-reactive protein attenuated nitric oxide production in endothelial cells and inhibited angiogenesis [403]. Furthermore, Bodman-Smith and colleagues [23], showed that affinity purified CRP from acute phase serum did not affect the production of pro-inflammatory cytokine production by U937 human macrophage-like cells, and did not alter *Leishmania donovani* survival in macrophages [386]. My findings indicate that recombinant goldfish CRP produced in *E. coli* did not induce pro-inflammatory functional responses in goldfish phagocytes, since the production of reactive oxygen and nitrogen intermediates in monocytes and macrophages, respectively was similar between CRP-treated and non-treated cells (Fig 5.7 and 5.8). Lack of a significant NO responses may be due to several evasions mechanisms. *T. carassii* gp63 has been shown to reduce macrophage antimicrobial responses [312]. In addition, *T. carassii* CRT may be able to bind CRP and affect NO production [9]. *T. carassii* possess redundant moieties that interact with effector molecules and immune cells allowing it to establish [272].

It has been suggested that the conformation of CRP determines the inflammatory profile [111], and can be attributed to the controversial findings regarding CRP function. The dissociation of the native pentameric CRP (pCRP) can be mediated by cells in sites of inflammation resulting in the monomeric or modified isoform CRP (mCRP). pCRP binds phosphocoline and others effector molecules such as C1q [404] whereas the mCRP has pro-inflammatory effects by its ability to bind integrins on cells such as macrophages and neutrophils [405]. Both isoforms have been documented to interact with C1q,

activating the classical pathway of complement [399]. However, the functional aspects the properties of both CRP isoforms require further research.

In *T. carassii* infections CRP may play a role in modulating complement, however unlike mammalian counterparts, rgfCRP does not activate antimicrobial effector responses in goldfish immune populations in response to *T. carassii*.

```
1      ACAGGGGACCCCATCCCTAATCTAATAGCGCATCTGTCATATAAGATCTAGTAGACAT      58
59   TACCTGAAGCatgatgctgatgccagtggtttcatttttctgtctgctctctccgactcta   119
                M  M  L  M  P  V  V  S  F  F  C  L  L  S  P  T  L
120    gcgattactgaagtgggcctcagtggtaacctgcttctgtttccaatcaagactaacacc   179
        A  I  T  E  V  G  L  S  G  N  L  L  L  F  P  I  K  T  N  T
180    agctttgtcaaactccttcctgaagagccattgagtctttcagcgtttactctctgcatg   239
        S  F  V  K  L  L  P  E  E  P  L  S  L  S  A  F  T  L  C  M
240    cgtgtggcgacggagctccagggtgaccgggagatcattctgttcgcttaccgcacgccc   299
        R  V  A  T  E  L  Q  G  D  R  E  I  I  L  F  A  Y  R  T  P
300    gagtttgatgaactcaacgtgtggagacgtgacgatggttgtgtggccttggtaatccag   359
        E  F  D  E  L  N  V  W  R  R  D  D  G  C  V  A  L  V  I  Q
360    tctaatggcgaagaagcatttttccatctgcctcctctctccaccttccagactcacctg   419
        S  N  G  E  E  A  F  F  H  L  P  P  L  S  T  F  Q  T  H  L
420    tgtgtcacctggagctctgcgtctggtctcactgccttctgggtggatggacgtcgcagt   479
        C  V  T  W  S  S  A  S  G  L  T  A  F  W  V  D  G  R  R  S
480    tcgttccagatctatagaaaaggttactcaatccgtcctggtggcaccgtcgtgctcggc   539
        S  F  Q  I  Y  R  K  G  Y  S  I  R  P  G  G  T  V  V  L  G
540    caggaccctgatagatatctgggtgcctttaacgcagagcagagttttgtaggagaaatt   599
        Q  D  P  D  R  Y  L  G  A  F  N  A  E  Q  S  F  V  G  E  I
600    tcagatgtgcaaatgtgggactatgttctccctggcagtcagattaaggcggtttattca   659
        S  D  V  Q  M  W  D  Y  V  L  P  G  S  Q  I  K  A  V  Y  S
660    aaccaggaaccgtatgtgccaaagggaaacgtgttcgactggaacaccgtggaatatgag   719
        N  Q  E  P  Y  V  P  K  G  N  V  F  D  W  N  T  V  E  Y  E
720    attaatggaaatgtgctagtggtgcaaattaactgaTTCTGATCAGTTGTCACGTCTCAC   779
        I  N  G  N  V  L  V  V  Q  I  N  *
780    ACCGGCTCATCCACGCAGTTATGTCAATTTCTATAATCGATTCATCTGTTTTAATGATTT   839
840    TCAAAACTGTAAGGGCCAACAAGTAGGAATTGTTCACAAAAGTAACCAGTAAAAGCTCTC   899
900    TGCATTACAAAAAAAAAAAAAAAAAAAAAAAAAAA                            935
```

Figure 5.1 Goldfish CRP full-length sequence showing ORF, untranslated regions (UTR) and various domains. At the 5' and 3' ends, the UTR is shown in upper case letters. The open reading frame (ORF) is in lower case letters, start and stop codons are double-underlined. The pentraxin domain is marked with grey. The signal peptide is underlined.

```
AsianSeabass      MRLSAVVLLIS----TS--LVLAGSVVIKTMVFPIETSTSYVELVPLKPLDLRAFTLCMR  54
Zebrafish         -----MLLFFCLLSVTLEATEGFKNLSGKVLQFKTATDNSYVKLYPEKPLSLSAFTLCMR  55
Grasscarp         ----MLVLFFCLLS-LTA-AAAEVGLGGKVLLFPNKTDSSYVKLTPEKSLSLKAFTLCMR  54
Goldfish          MMLMPVVSFFCLLS-PTL-AITEVGLSGNLLLFPIKTNTSFVKLLPEEPLSLSAFTLCMR  58
Commoncarp        MMLVPVVLFFCLLLSLTA-AATEVGLVGKVLLFPTKTNTSFVALTPEKPLSLSAFTLCMR  59
                       :: ::.              :  : : *   *..*:* * * : *.* *******

AsianSeabass      VATELKGEREVILLAYRTEDHDELNVWRELDGRLSLYLASSGESVLFEVPQLEALETHLC  114
Zebrafish         VATELPLDREVILFAYYTPDVDELNVWRERDGRVSLYIQSSKDAAFFRLPPLSTLQTHLC  115
Grasscarp         VAMELQSEREIILFAYCMPGFDELNVWREKDGRVSLYLHSSGEAAFFHLPPLSIFHTHMC  114
Goldfish          VATELQGDREIILFAYRTPEFDELNVWRRDDGCVALVIQSNGEEAFFHLPPLSTFQTHLC  118
Commoncarp        VATELQGERETILFAYRTQDYDELNVWREKDGRLSFYL--SGSGAFFNLPALSTFGTHLC  117
                  ** **  :** **:**     *******. ** ::: :  . . .:*.:* *. : **:*

AsianSeabass      VTWDSSSGAAALFMNGRKSLTKIYKKDHAIRSGGKVIIGQDPDNFLGGFDQKQCFIGEIS  174
Zebrafish         VAWESATGLTAFWMDGRRSLHQVYRKGYSIRSGGTVVLGQDPDSYVGSFDVDQSFVGEIA  175
Grasscarp         VTWDSAPGLAAFWVDGRRSSFQLYRKGHSVLPGGTILLGQDADSCPGSFDAEQSFVGEIT  174
Goldfish          VTWSSASGLTAFWVDGRRSSFQIYRKGYSIRPGGTVVLGQDPDRYLGAFNAEQSFVGEIS  178
Commoncarp        LTWDSETGLSAFWMNGHRSTFQLYRKGHSIRPGGTVLLGQDPDNYLGAFEVEQSFVGEIT  177
                  ::*.*  * :*::::*::*  ::*:* :::  **.:::*** *   *.*: .*.*:***:

AsianSeabass      DVNMWDSVLSDSTIQDMYAG--KRVPRGNVFDWENTELKINGEVEVITREL   223
Zebrafish         NLQMWDYVLSSAQIKAVYYNQDNR-VKGNVFDWDTIEYDVTGNVLVVPDN-   224
Grasscarp         DVKMWNYVLSGSQIKAVYSNQEPYVPKGNVFDWSTIKYETRGNVLVVENN-   224
Goldfish          DVQMWDYVLPGSQIKAVYSNQEPYVPKGNVFDWNTVEYEINGNVLVVQIN-   228
Commoncarp        DVHMWDHVLSGSQIMAVYSNQEPYVPKGNVFDWNTIKYEINGSVLVVQES-   227
                  ::.**: **  : *  :*        :******.. : .  *.* *:  .
```

Figure 5.2 Protein alignment of goldfish CRP with other known fish sequences. Amino acid alignment of goldfish CRP, zebrafish CRP, Asian Seabass CRP, common carp PTX and grasscarp PTX. Fully conserved amino acids residues appearing in all sequences are indicated with a star (*), amino acids that have strong conservation are indicated with (:) and weak conservation is indicated with (.).

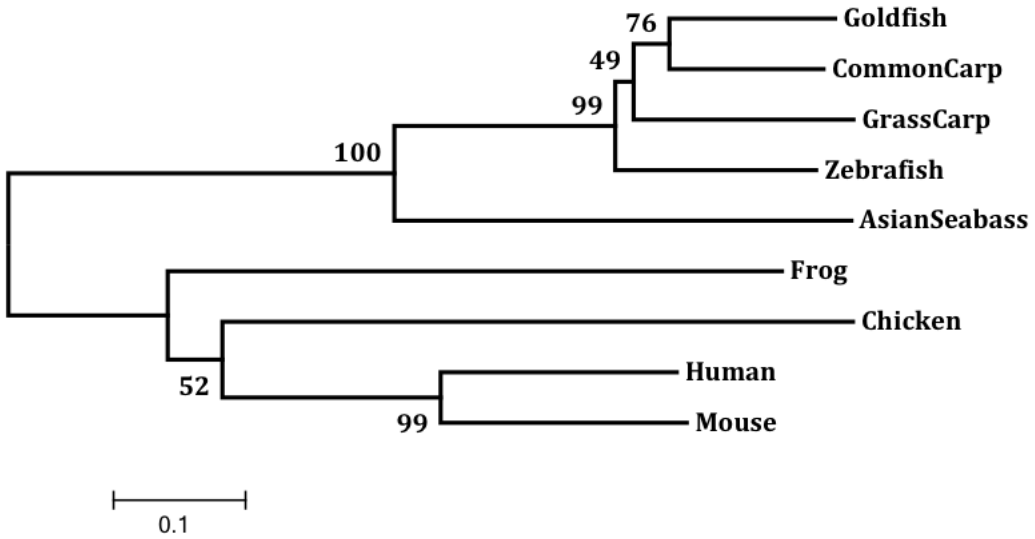

Figure 5.3 Phylogenetic analysis of goldfish CRP against CRP of other known isoforms in vertebrates. Amino acid sequences were aligned by using CLUSTAL-W program and an unrooted phylogenetic tree was constructed using the neighbor-joining method of the MEGA 5 software. The tree was bootstrapped 10,000 times. Nucleotide and residue sequences used here were obtained from GenBank database (accession no.): common carp CRP-like (AEU04519.1), grass carp PTX (ACM04449.1), zebrafish CRP (AGB69035.1), rainbow trout CRP-PTX related (NP_001118197.1), Asian sebass CRP (ADX06859.1), human CRP (ADX06859.1), mouse CRP (CAA31928.1), frog CRP (AAA49692.1) and chicken CRP (ABD16281.1).

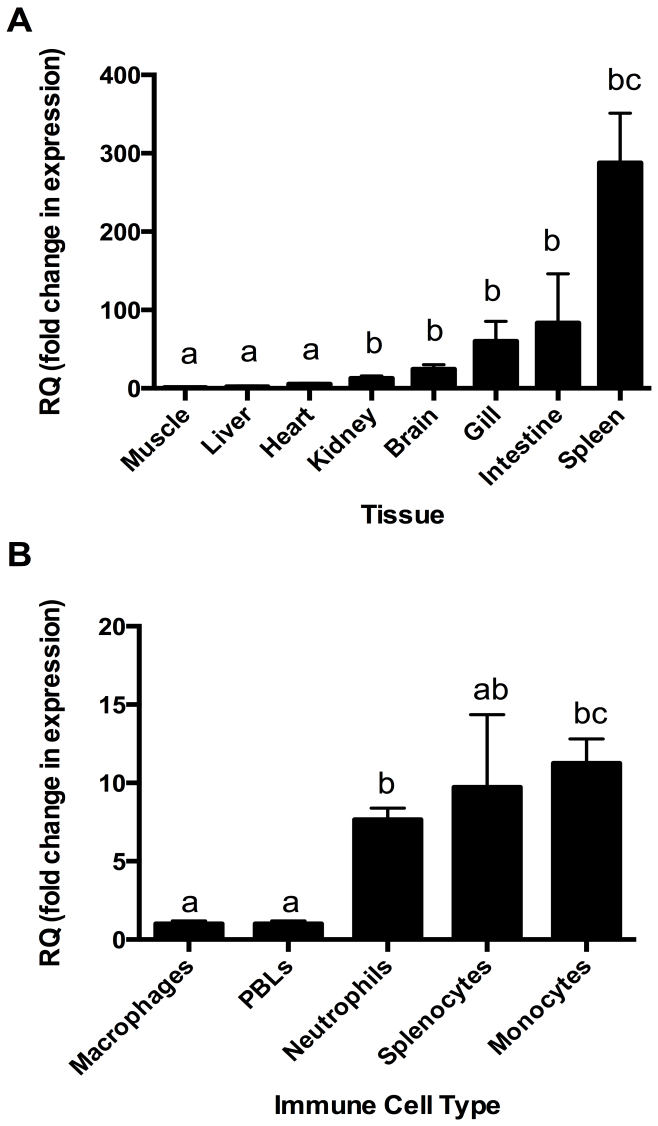

Figure 5.4 Quantitative constitutive expression analyses of goldfish CRP expression in goldfish tissues and immune cell populations obtained from normal fish. Goldfish CRP expression in goldfish tissue and immune cell populations is relative to endogenous control gene, elongation factor 1 alpha (EF-1α). The mean Ct value for EF-1α was in the range of 21.54 - 21.8 and 19.34-19.76 for all immune cell populations and tissues, respectively. **(A)** Goldfish CRP tissue analysis. Analysis of the relative expression data are pooled from 5 individual, performed in triplicate, and normalized against muscle. **(B)** Goldfish CRP expression in non-stimulated goldfish immune cells. The cells examined were: monocytes, macrophages, peripheral blood leukocytes (PBL), neutrophils and splenocytes. Analysis of the relative expression data are pooled from 5 individual, performed in triplicate, and normalized against FACS-sorted macrophages for immune cell distribution. Statistical analysis was performed using one-way followed by Dunnett's post hoc test. Different letters above each bar denote statistically different ($P < 0.05$), and the same letter indicates no statistical differences between groups.

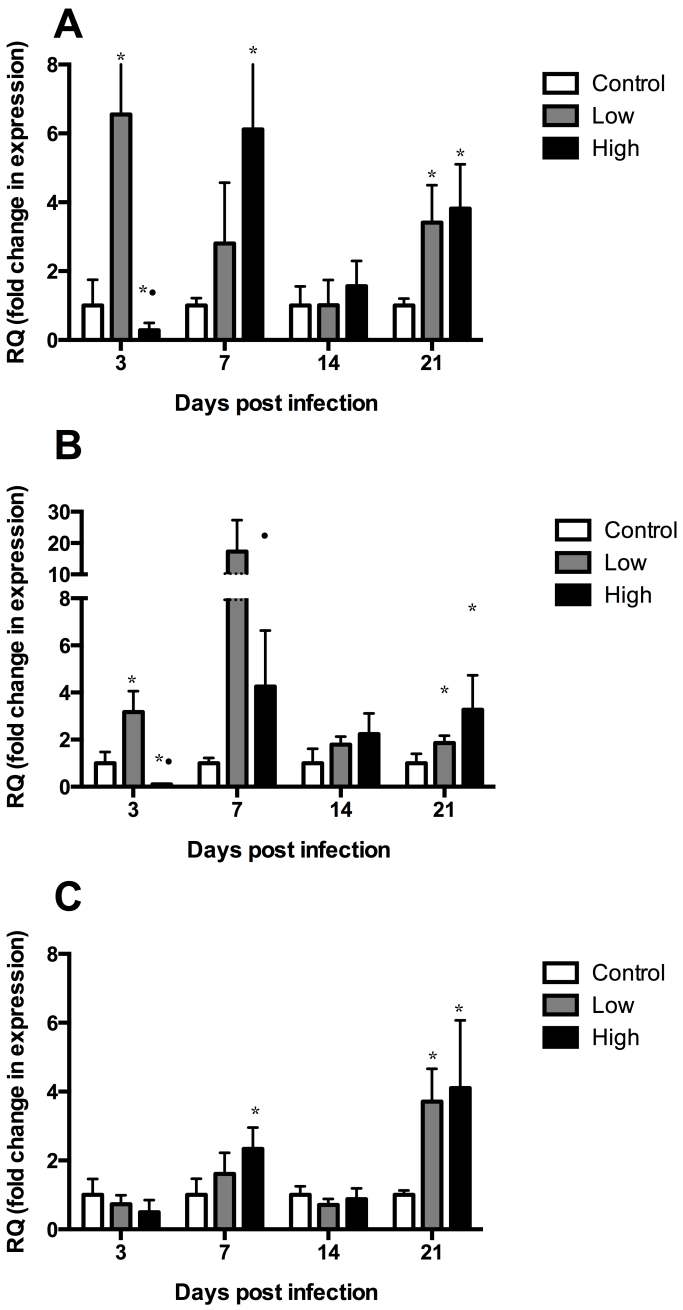

Figure 5.5 Quantitative expression analyses of goldfish CRP expression of goldfish infected with two doses of *T.carassii* and non-infected goldfish (A) Goldfish CRP kidney **(B)** Goldfish CRP liver **(C)** Goldfish CRP spleen. Expression was relative to endogenous control gene, elongation factor 1 alpha (EF-1α) and normalized against those observed in non-infected control. Data are represented as mean ± SEM of 6 fish ($P < 0.05$). Asterisks (*) denote statistical significance from non-infected control fish.

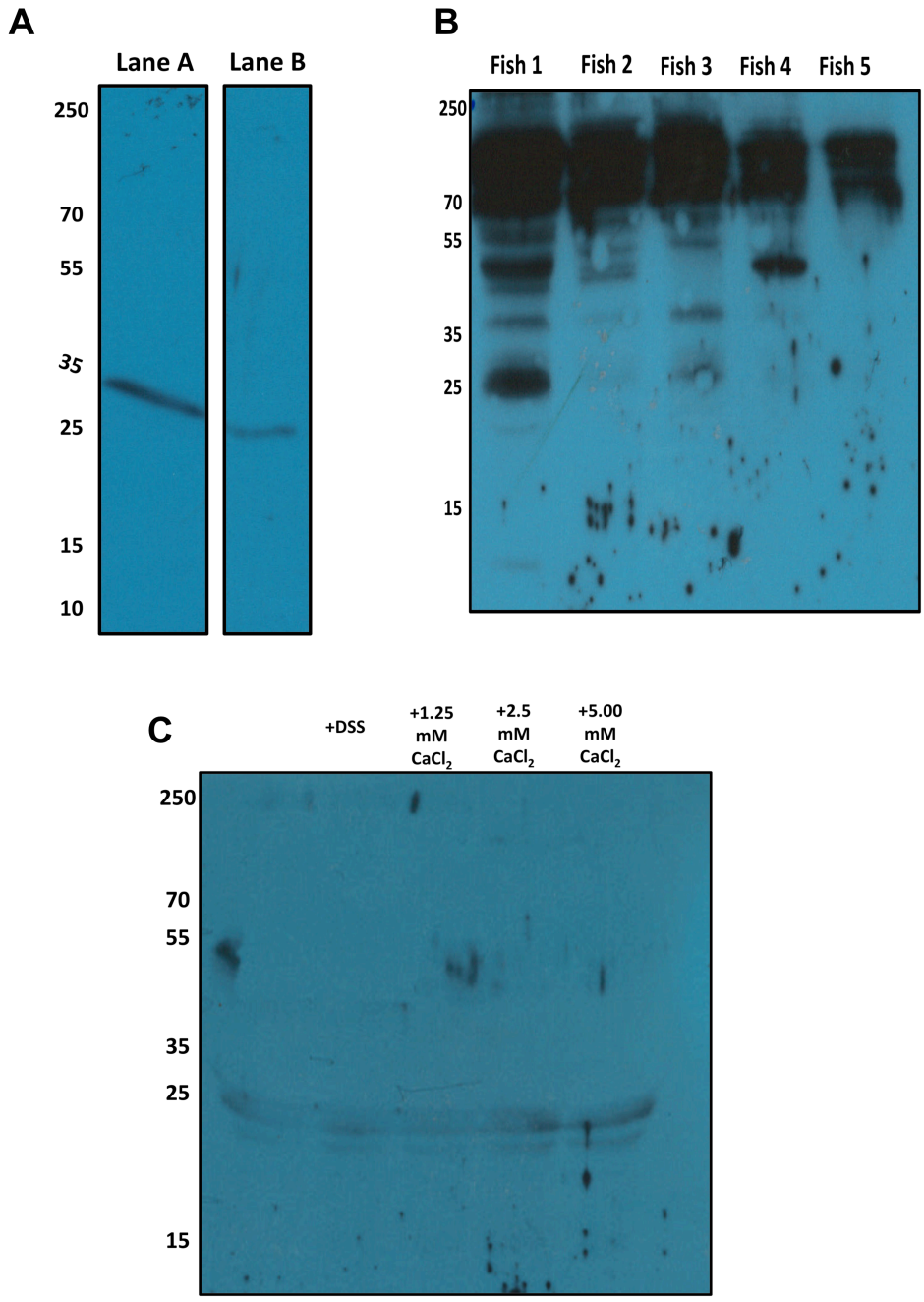

Figure 5.6 Western blot analysis of recombinant goldfish CRP. (A) Analysis of recombinant rgfCRP Lane A: Un-cleaved rgfCRP visualized using mouse anti-histidine monoclonal antibody (1:5000) Lane B: Cleaved rgfCRP visualized using rabbit anti-rgfCRP purified IgG polyclonal antibody (1:3000) raised against rgfCRP. **(B) Analysis of CRP in sera from *T. carassii* infected goldfish** visualized using rabbit anti-rgfCRP purified IgG polyclonal antibody (1:3000) raised against rgfCRP. **(C) *In vitro* binding assessment of rgfCRP** using DSS and various concentrations of $CaCl_2$.

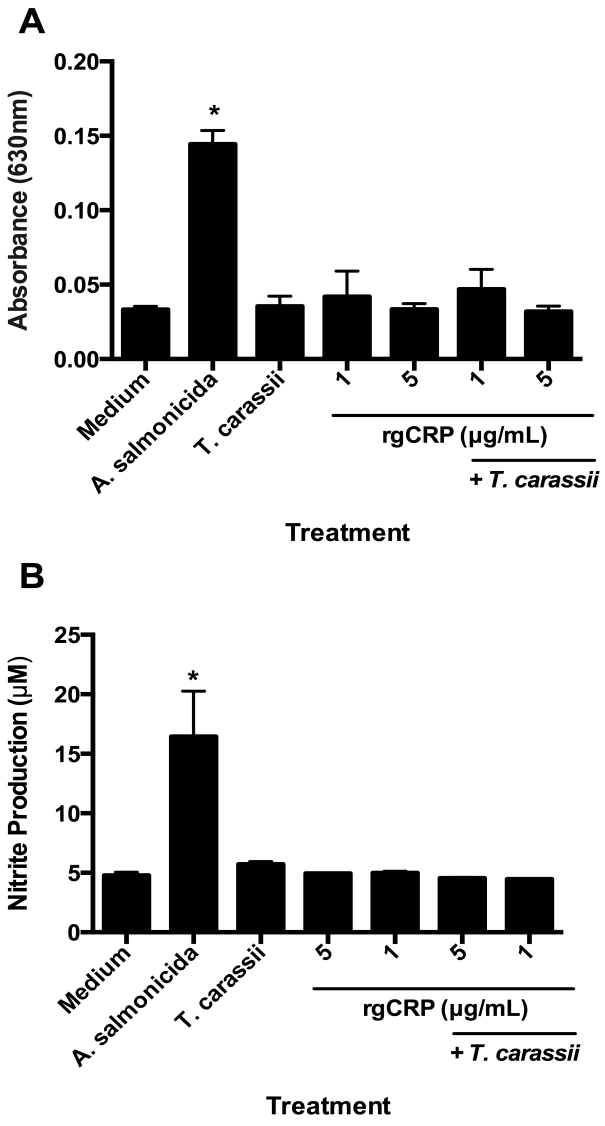

Figure 5.7 Cleaved recombinant goldfish CRP (rgfCRP) does not induce the production of reactive oxygen and nitrogen intermediates in monocytes and macrophages exposed to *T. carassii*: (A) rgfCRP ability to enhance respiratory burst against *T. carassii*. The absorbance values of medium-treated controls (no PMA) were subtracted from treatment values to factor in background NBT reduction. Data are represented as mean ± SEM of relative reactive oxygen intermediate production by monocytes ($n = 3$). (B) rgfCRP ability to enhance nitrite production in goldfish macrophages against *T. carassii*. Nitric oxide production was determined using the Griess reaction and nitrite concentration was deduced using a nitrite standard curve. Data are represented as mean ± SEM nitrite production by macrophages from separate cultures established from three individual fish ($n = 3$). Statistical analysis was done using one-way ANOVA with a Tukey's multiple comparison tests. Asterisk (*) denotes statistically different ($P < 0.05$) from medium control.

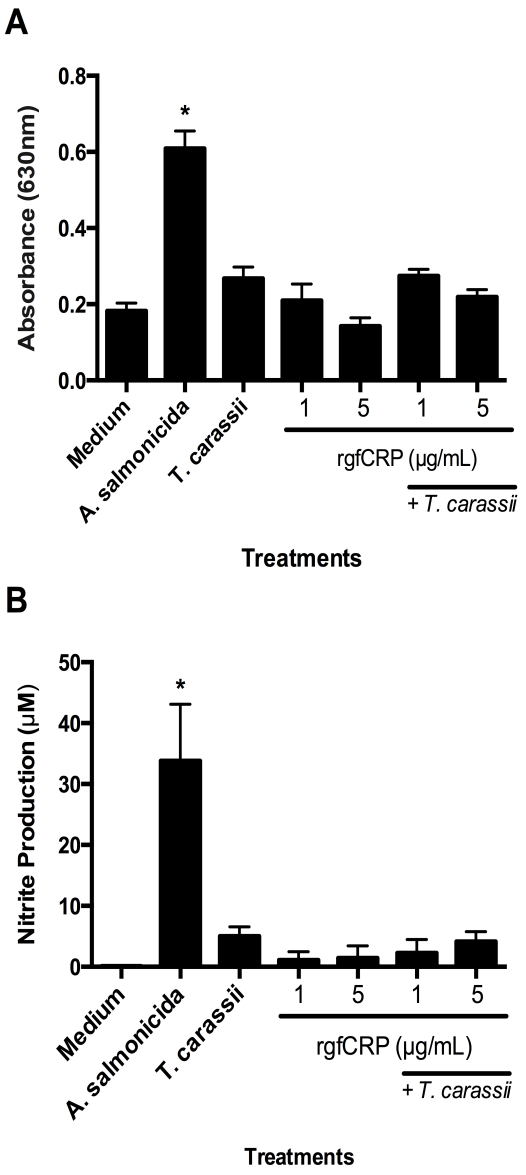

Figure 5.8 Un-cleaved recombinant goldfish CRP (rgfCRP) does not induce the production of reactive oxygen and nitrogen intermediates in monocytes and macrophages exposed to *T. carassii*: (A) rgfCRP ability to enhance respiratory burst against *T. carassii*. The absorbance values of medium-treated controls (no PMA) were subtracted from treatment values to factor in background NBT reduction. Data are represented as mean ± SEM of relative reactive oxygen intermediate production by monocytes ($n = 3$). (B) rgfCRP ability to enhance nitrite production in goldfish macrophages against *T. carassii*. Nitric oxide production was determined using the Griess reaction and nitrite concentration was deduced using a nitrite standard curve. Data are represented as mean ± SEM nitrite production by macrophages from separate cultures established from three individual fish ($n = 3$). Statistical analysis was done using one-way ANOVA with a Tukey's multiple comparison tests. Asterisk (*) denotes statistically different ($P < 0.05$) from medium control.

Table 5.1 Parasitemia in goldfish infected with two doses of *Trypanosoma carassii*[1]

Days Post-Infection	Number of Trypanosomes/mL (log_{10}) (Mean ± SEM)	
	Low Dose	**High Dose**
3	4.91 ± 0.48	5.00 ± 0.24
7	5.25 ± 0.43	5.92 ± 0.49
14	6.16 ± 0.35	6.53 ± 0.25
21	5.92 ± 0.25	6.81 ± 0.12 *

[1]Fish (n=6) were inoculated with low (6.25×10^6) or high (1×10^7) doses of *in vitro* growth *T. carassii.* Blood samples were collected for individual fish at 3, 7, 14 and 21 days post infection and the number of parasites enumerated. Results are displayed as mean of parasites per mL of blood ± SEM. Asterisks (*) denote statistical significance between two doses of trypanosome infection groups ($P < 0.05$).

Table 5.2 Lysis of normal and trypsin treated *Trypanosoma carassii* by sera from infected and uninfected fish and addition of recombinant goldfish C-reactive protein.[1]

Parasite Treatment	Serum Type	Number of trypanosomes per well (log_{10}) (Mean ± SEM)		
		PBS	0.5 μg/mL	5 μg/mL
None	Medium	4.99 ± 0.03	5.00 ± 0.02	4.98 ± 0.04
	HI non-immune	4.92 ± 0.03	4.92 ± 0.04	4.90 ± 0.02
	Non-immune	4.91 ± 0.02	4.90 ± 0.01	4.68 ± 0.01 *
	Immune	4.90 ± 0.02	4.75 ± 0.07 *	4.48 ± 0.11 *
Trypsin	Medium	4.92 ± 0.03	4.92 ± 0.05	4.88 ± 0.04
	HI non-immune	4.90 ± 0.02	4.87 ± 0.03	4.93 ± 0.04
	Non-immune	0 *	0 *	0 *
	Immune	0 *	0 *	0 *

[1]Control and trypsin-treated cultured trypanosomes were exposed to serum from both infected and uninfected goldfish with either medium, 0.5 μg/mL or 5 μg/mL rgfCRP as described in methods for 1 hour at 20°C. HI denotes heat-inactivated. Immune serum refers to pooled serum collected from 6 individual fish 42 days post infection with *T. carassii.* Medium refers to trypanosome culture medium containing 10% heat-inactivated goldfish serum. Data are represented as mean number of parasites per well (log_{10}) ± SEM, calculated from duplicate wells from three replicate experiments. Asterisk (*) denotes significant difference ($P < 0.05$) in Bonferroni-corrected pairwise comparisons between protein treatment for each serum type.

Table 5.3 Recombinant goldfish CRP does not act as an opsonin to enhance phagocytosis of *T. carassii* or *E.coli* in goldfish macrophages.[1]

Sample	Percent Phagocytosis (Mean ± SEM)	
	Internalized	Surface bound
E.coli DH5α-GFP	17.43 ± 3.17	23.43 ± 4.29
E.coli DH5α-GFP + 0.5μg/mL rgfCRP	17.47 ± 0.26	24.11 ± 0.63
E.coli DH5α-GFP + 5μg/mL rgfCRP	17.27 ± 2.09	22.36 ± 1.61
T. carassii-FITC	0	0
T. carassii-FITC + 0.5μg/mL rgfCRP	0	0
T. carassii-FITC+ 5μg/mL rgfCRP	0	0

[1]*E.coli* DH5α-GFP or *T.carassii*-FITC were opsonized for 30 minutes with or without the addition of varying concentration of recombinant goldfish CRP. Samples were then incubated with goldfish macrophages (n=2) for 2 hours, after which cells were fixed and phagocytosis was analyzed using ImageStream flow cytometer.

CHAPTER 6

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF GOLDFISH SERUM AMYLOID A (SAA)[1]

6.0 Introduction

Serum Amyloid A (SAA) is a major acute phase protein, whose serum levels can increase 1000-fold in humans during the acute phase response (APR) in response to various insults [125]. It is primarily synthesized in the liver and its production is regulated by cytokines such as interleukin-6 (IL-6), IL-1, and tumor necrosis factor-alpha (TNFα) [80].

The SAA family is composed of acute phase and constitutive proteins. Acute phase SAA (A-SAA) is induced by inflammatory cues and constitutive SAA (C-SAA) is constitutively expressed [133]. C-SAA is present in humans and mice only, whereas A-SAA has been documented in all vertebrates [125]. In humans, A-SAA is encoded by 2 genes, and in mice 3 genes, respectively. In invertebrates such as echinoderms and lower vertebrates such as teleosts, there is a single identified SAA gene, suggesting that SAA multi-gene family only evolved in mammals [136,137]. The presence of SAA dates to echinoderms, and based on the high amino acid conservation throughout 500 million years of evolution, and lack of SAA-deficient animals, SAA is vital to survival [124,130]. Despite the importance of SAA for well being of organisms, its functions remains to be fully elucidated, particularly in lower vertebrates.

In humans, SAA appears to have cytokine-like properties [138]. The addition of SAA induces production of cytokines [128,129], chemokines [139] and matrix metalloproteinases (MMPs) [140] in human monocytes. SAA has been demonstrated to act as a chemoattractant for phagocytes, T lymphocytes, and granulocytes (neutrophils) [141–143]. SAA has been shown to bind to a diverse group of receptors, including TLR2 [127], TLR4 [144] formyl peptide receptor-like 1 (FPRL1) [145] and CD36 [146]. Given this, it is not surprising that the physiological effects of SAA are pleiotropic. For example, SAA acts as a priming agent of neutrophils [406], induces production of cytokines such

[1]A version of this chapter is in review: Kovacevic, N., and Belosevic, M. 2015. Molecular and functional characterization of goldfish (*Carassius auratus* L.) Serum Amyloid A. *Fish and Shellfish Immnunology* (in review)

as TNFα, IL-1β in immune cells [128,129,365] and antimicrobial effector functions [145]. SAA has also been reported to have anti-inflammatory effects, such as inhibition of oxidative burst in neutrophils [407,408] and stimulation of production of anti-inflammatory cytokines such as IL-10 [128,129].

In fish SAA was first identified in salmonids [149]. Thereafter, it has been identified in several bony fish species including carp (*Cyprinus caprio)* [150], rainbow trout (*Oncorhynchus mykiss)* [136], zebrafish *(Danio rerio)* [151] Atlantic cod (*Gadus morhua* L.) [367] (*Epinephelus coioides* [409] Asian Seabass (*Lates calcarifer)* [152], and rock bream (*Oplegnathus fasciatus)* [410].

Up-regulation of SAA has been observed in various fish species in response to numerous chemical stimuli and pathogens, re-enforcing a consensus that SAA may play an important role in immunomodulation. However, there is limited research exploring the functional role of SAA in fish. In this chapter, I report on studies whose aim was to clone, express and functionally characterize goldfish recombinant SAA. Goldfish SAA mRNA levels were measured in normal goldfish tissues, and in different immune cell populations. The gene expression of pro- and anti-inflammatory cytokines was examined in monocytes and macrophages stimulated with recombinant goldfish SAA (rgSAA). Additionally, I examined whether the rgSAA modulated antimicrobial responses in cultured goldfish monocytes and macrophages.

6.1. Experimental Design

6.1.1 Molecular cloning and expression of rgSAA

Primers were designed based on the nucleotide sequences of the SAA genes of common carp (Accession No. AB016524.1), and zebrafish (Accession No. NM_001005599.1). RACE PCR (Clonetech, USA) was performed to obtain a full open reading frame and untranslated 5’ and 3’ sequences. Sequences were obtained as described in Section 3.3.

6.1.2 Quantitative expression of SAA in non-infected, low *T. carassii* infected and high *T. carassii* infected goldfish during the acute infection

Fish were anaesthetized with TMS and were injected intraperitoneally with 6.25×10^6 or 1×10^7 *in vitro* grown parasites suspended in 100 μL of TDL-15 medium, or sham injected with 100 μL of TDL-15 (control fish). Collection and enumeration of parasitemia was accomplished as previously described [281,355] and in Section 3.1.6. Number of parasites per mL was log transformed and presented as mean ± SEM parasites per mL of blood of (n=6) fish per experimental group.

6.1.3 Quantitative expression of SAA in normal goldfish tissues and non-stimulated immune cell populations

Isolation of different immune populations was carried out as described in Chapter 3. Preparation of cDNA from goldfish tissues and immune cell population and quantitative thermocycling conditions were previously described [382]. Briefly, total RNA was extracted using TRIzol and reverse transcribed into cDNA using Superscript III cDNA synthesis kit (Invitrogen) according to manufacturer's directions. Expression of SAA was assessed relative to the endogenous control, elongation factor 1 alpha (EF-1α). Tissue and immune cell populations were obtained from individual goldfish (n=5), and used in quantitative PCR analysis using 7500 Fast Software (Applied Biosystems). The RQ values were normalized against the lowest expression observed in tissue (muscle) and immune cell population (macrophage).

6.1.4 Generation of recombinant goldfish SAA (rgSAA) using prokaryotic expression

The production and generation of recombinant goldfish SAA was carried out as described in Section 3.4.2 and 3.4.3. Briefly, the PCR fragment encoding the mature, signal sequence cleaved goldfish SAA was amplified using primers that allowed cloning into pET SUMO expression vector (Invitrogen). Correct in-frame insert was transformed into BL21 (DE3) One Shot *E. coli* cells (Invitrogen), scaled up and induced according to a pilot study. Purification was carried out using MagneHis Ni-particles (Promega) and a

PolyATtrack System 1000 magnet (Promega) under denaturing conditions. The endotoxin was removed from the recombinant protein using Pierce High Capacity Endotoxin Columns (Thermo Scientific) according to manufacturer's directions. Recombinant protein was analyzed using the *Limulus* amebocyte lysate kit (Thermo Scientific) and found to be endotoxin free. Protein concentration was determined using a Micro BCA Protein Assay Kit (Thermo Scientific).

6.1.5 Assessment of rgSAA to act as a chemotactic factor in goldfish immune populations

Chemotactic activity was assessed as previously described [60]. Immune cells from four individual fish cultures (kidney-derived neutrophils, monocytes and mature macrophages) were washed twice in NMGFL-15 medium and adjusted to a final concentration of 1 x 10^6 cells/mL. The chemotaxis assay was performed using blind well leucite chemotaxis chambers (Nucleoprobe Corp.) using a 5 μm pore size polycarbonate membrane filters (Neuroprobe) to separate the top and bottom chambers. 100 μL of the rgSAA (0.1, 1, and 10 μg/mL) and 100 μL of NMGFL-15 were added to the bottom well of the chemotaxis chambers. To the top chamber, 100 μL of cell suspension was added plus 100 μL of medium. Negative controls consisted of medium alone and the positive control was 20 ng/mL fMLP (N-formyl-methionyl-leucyl-phenylalanine). The chemokinesis control consisted of equal amounts of the treatment in both the upper and lower chambers. The incubation was 1 hour for neutrophils and 4 hours for PKMs, after which the cell suspension was aspirated from the top well, and the filter removed. Filters were fixed in methanol, rinsed and stained with Gill's Solution 3 (Sigma) to visualize cells. Chemotactic activity was assessed by counting the total number of cells in 20 random fields of view under oil immersion (100X).

6.1.6 Assessment of the ability of recombinant SAA to induce mRNA levels of genes encoding cytokines in monocytes and macrophages

Monocytes and macrophages were obtained from individual fish (n=4) as described above and seeded into individual wells of a 24-well plate at a density of 1 x 10^6 cells per well in a final volume of 500 μL CMGFL-15. Cells were treated with PBS, 0.5 or 5 μg/mL rgSAA for 1, 3, 6, and 12 hours. Total RNA was isolated from cells at indicated times using TRIzol Reagant (Invitrogen) and reverse transcribed into cDNA as

described previously. Changes in the gene expression of pro-inflammatory and anti-inflammatory cytokines were examined (Table 3.7). Expression was relative to EF-1α and RQ values were normalized against respective untreated controls.

6.1.7 Assessment of the effects of rgSAA on respiratory burst of monocytes

Respiratory burst assay was performed as previously described [383]. Briefly, monocytes from individual fish (n=5) were seeded into 96-well plates at a density of 3×10^5 cells/well and incubated in culture medium alone (control) or pre-treated with one of the following for 3 hours: rgSAA (0.01, 0.1, 1, and 10 μg/mL), with subsequent addition of heat-killed (HK) *A. salmonicida*, and incubated for 18 hours at 20°C after which NBT (2 mg/mL, Sigma) and PMA (final triggering concentration 100 ng/mL; Sigma) in PBS were added to the cultures and incubated at room temperature for 30 minutes. Production of ROI was determined as described in Section 3.6.1.

6.1.8 Assessment of the effects of rgSAA on nitric oxide production of macrophages

Nitric oxide (NO) assay was performed as previously described [383]. Briefly, mature macrophages from individual fish (n=6) were seeded in wells of 96-well plates at a density 3×10^5 cells/well. These were then incubated in culture medium alone (control) or treated with one of the following: rgSAA (0.05, 0.1, 0.5, 1, 5 and 10 μg/mL) with or without HK-*A. salmonicida*, and incubated at 20°C for 72 h. Nitrite production was determined as described in Section 3.6.2.

6.1.9 Assessment of the effects of rgSAA on phagocytosis of neutrophils and macrophages

Mature macrophages or kidney-derived neutrophils (n=3) were either: pre-treated with varying concentrations or rgSAA (0.01, 0.1, 1, and 10 μg/mL) and *E.coli* DH5α-GFP subsequently added or *E.coli* DH5α-GFP was opsonized with varying concentrations of rgSAA (0.01, 0.1, 1, and 10 μg/mL) or a PBS control for 30 minutes, after which it was added to immune cells. Incubation, collection and analysis of data are described in Section 3.6.4.3.

6.1.10 Assessment of direct antibacterial function of rgSAA

A growth curve of *E.coli* DH5α-GFP was generated correlating to A_{600} in conjunction with the number of colony forming units (CFUs) after plating bacterial serial dilutions. 1 x 10^6 bacteria were washed 3X in PBS and various concentrations of rgSAA (0.01, 0.1, 1, and 10 μg/mL) were added and incubated for 2 hours at 37°C. Bacteria with PBS served as a control to determine 100% viability. To ascertain the results were not due to preparation procedures, rgfCRP subject to the same purification procedures was used as a control [382]. After incubation, serial dilutions were spread on LB-Ampicillin to achieve suitable amount of bacteria for counting. The experiment was performed 5 times (n=5) with duplicate plates for each treatment.

6.2 Results

6.2.1 Goldfish SAA is similar to SAA of other teleosts

The complete open reading frames (ORFs) and the untranslated regions (UTR) of the goldfish SAA cDNA transcripts was obtained. Goldfish SAA is 660 base pairs long, with an ORF encoding a 123 amino acid protein with a predicted size of ~13 kDa (Fig. 6.1). The nucleotide sequences of goldfish SAA (Accession No. KT598223) has been submitted to the Genbank.

Protein alignment of goldfish SAA with other known vertebrae sequences indicated that goldfish SAA amino acid sequence was highly conserved among teleosts (Fig. 6.2). The sequence contained all basic elements characteristic of SAA superfamily. This included the SAA family signature A-R-G-N-Y-[ED]-A-x-[QKR]-R-G-x-G-G-x-W-A, a 'GPGG' calcium binding domain and 'RGN' fibronectin-like binding domain. All SAA protein sequences contained a signal peptide, in addition to a serum amyloid domain in similar position. Furthermore, goldfish SAA displayed high intrinsic folding indicative of its reactivity, which was similar to all SAA sequences examined (Table 6.2).

To assess the phylogenic characteristics of SAA, an unrooted phylogenic tree was constructed using goldfish SAA and known SAAs from other fish and higher vertebrates. Goldfish SAA was more closely related to SAAs of other cyprinifome species, and

branched separately from perciformes SAAs (Fig. 6.3). All fish SAAs branched separately from mammalian SAAs (Fig. 6.3).

6.2.2 SAA gene expression in goldfish infected with low and high dose of *T. carassii*

The course of infection was monitored by measuring parasitemia (number of parasites/mL of blood) (Table 5.1). Parasitemia was highest at 14 dpi for the low dose *T. carassii* group, with a mean log burden of 6.16 ± 0.35 trypanosomes/mL, after which parasite load began to decline. In contrast, in the high dose *T. carassii* group, parasite numbers continued to increase, reaching a peak at 21 dpi (6.81 ± 0.12 parasites/mL) (Table 5.1).

The mRNA levels of SAA were significantly up-regulated in the kidney in response to *T. carassii* infection, however, the mRNA levels in the high dose groups were significantly different from those of low dose groups on 7, 14 and 21 dpi (Fig. 6.4A). In the liver, expression of SAA mRNA levels of low and high *T. carassii* infected goldfish were significantly different from control counterparts, however there were no differences in SAA mRNA levels between low and high dose groups (Fig. 6.4B). Similar to the mRNA levels in the liver, SAA mRNA levels in both low and high dose groups were significantly up-regulated in infected compared to sham-injected non-infected control fish, the exception being the high dose group on 14 dpi (Fig. 6.4C).

6.2.3 SAA mRNA levels in the kidney and spleen, but not liver are related to parasitemia

SAA mRNA levels were found to be positively correlated with parasite load in the kidney (r=0.4513, p=0.0019) and the spleen (r=0.3747, p=0.0317) (Fig. 6.4 D,F). No statistically significant correlation between parasite load and SAA mRNA levels were observed in the liver (Fig. 6.4E).

6.2.4 Analysis of SAA expression in normal goldfish tissues and immune cell populations

Assessment of SAA in normal goldfish tissues was performed using muscle as a reference. Constitutive SAA mRNA levels were highest in kidney, spleen, and intestine, with lower expression levels observed in gill, heart, and liver (Fig. 6.5A).

The expression of goldfish SAA was assessed in different immune populations obtained from goldfish (Fig. 6.5B). The immune populations examined included monocytes, mature macrophages, splenocytes, kidney-derived neutrophils and peripheral blood leukocytes. Similar constitutive expression of SAA was observed in all immune cell populations with the exception of neutrophils, which displayed the highest mRNA levels of SAA (Fig. 6.5B).

6.2.5 Prokaryotic expression of recombinant goldfish SAA

With the aim to functionally characterize goldfish SAA, I expressed rgSAA in *E. coli* and purified it using a MagneHis protein purification system. A single band with the molecular weight of 26.5 kDA including the size of a ~13 kDA histidine tag was indicated by Western blot using anti-His antibody (Fig. 6.6).

6.2.6 Recombinant goldfish SAA induced chemotaxis of kidney-derived neutrophils and mature macrophages but not monocytes

Recombinant SAA did not induced chemotaxis in monocytes (Fig. 6.7A). In contrast, rgSAA induced a significant, concentration-dependent, chemotactic response in mature macrophages and kidney-derived neutrophils (Fig. 6.7B-C), with chemotaxis observed after addition of 1 and 10 μg/mL of rgSAA.

6.2.7 Recombinant SAA induces a differential expression profile of pro-inflammatory and anti-inflammatory cytokines in monocytes and macrophages

Previous studies have shown human recombinant SAA induces differential effects on monocyte and macrophages [128,129]. I examined whether rgSAA also induced different effects in goldfish monocytes and macrophages. The expression of pro-inflammatory and anti-inflammatory cytokines was determined in cultured primary monocytes (Fig. 6.8) and mature macrophages (Fig. 6.9) at 1, 3, 6 and 12 hours after addition of rgSAA.

Treatment with 0.5 and 5 μg/mL of rgSAA induced both pro-inflammatory and anti-inflammatory cytokine expression in monocytes in comparison to non-stimulated controls. Anti-inflammatory cytokine IL-10 mRNA levels significantly increased as well as pro-inflammatory cytokines TNFα1, TNFα2 at 3 hours post stimulation (Fig 6.8).

The addition of rgSAA to mature macrophage cultures, up-regulated the mRNA levels of IL-1β1, SAA, and IL-12p40 (Fig. 6.9). There were no changes in the expression of IL-12p35, IL-1β2 and inflammasome NLRP3 (Fig. 6.9).

In macrophages stimulated with 5 μg/mL rgSAA, there was a significant up-regulation of SAA mRNA levels as early as 3 hours post stimulation, and these elevated mRNA levels remained for 12 hours post stimulation, which was the end of the observation period (Fig. 6.9). In addition, up-regulation of IL-1β1 was observed as early as 1 hours post stimulation, but no changes were observed in IL-1β2 expression at any time point after treatment with rgSAA (Fig. 6.9).

6.2.8 Recombinant goldfish SAA modulation of monocyte and macrophage antimicrobial functions

To examine whether rgSAA influenced the ability of monocytes to produce reactive oxygen intermediates (ROI), the cell cultures were pre-treated with varying concentrations of rgSAA, before the monocytes were stimulated with heat-killed (HK) *A. salmonicida* and their ability produce ROI (= respiratory burst response) measured. As shown in Figure 6.10, treatment of monocytes with rgSAA did not induce respiratory burst response of monocytes.

To assess the ability of SAA to modulate antimicrobial responses of mature macrophages, nitrite production was measured after macrophages were activated with HK-*A. salmonicida*. When varying concentrations of rgSAA were added to non-activated macrophages, nitrite production was not induced, indicating that rgSAA alone does not induce this functional response of macrophages (Fig. 6.11). However, when rgSAA was added to macrophage cultures after macrophages were activated with HK-*A. salmonicida*, a concentration dependent effect was observed, where treatment with increasing amount of rgSAA suppressed the NO response of macrophages (Fig. 6.11). Statistical analysis revealed that both 5 and 10 μg/mL rgSAA treatments were statistically significant from the positive control (*A. salmonicida* only treatment) (Fig. 6.11).

6.2.9 Recombinant goldfish SAA does not act as an opsonin to enhance phagocytosis of *E. coli* in neutrophils and macrophages

Human recombinant SAA has been shown to enhance phagocytosis [411], therefore I examined whether goldfish SAA exhibited similar properties. Surprisingly, the opsonization of *E. coli* with varying concentrations of rgSAA resulted in an apparent reduction in phagocytosis in both macrophages and neutrophils (Table 6.3). However, if cells are pre-treated with rgSAA with subsequent addition of *E. coli*, no significant changes in phagocytosis were observed (Table 6.4).

6.2.10 Assessment of antibacterial properties of SAA

Due to the confounding findings of rgSAA on phagocytosis, I wanted to assess whether rgSAA was acting on the bacteria alone, since pre-treatment did not change the phagocytic capacity of the cells. The addition of increasing concentrations of rgSAA caused a significant reduction in the number of viable bacteria, with highest reduction (30%) observed when 10 μg/mL rgSAA was added to bacterial cultures (Fig. 6.12A-B). Bacterial viability was correlated to rgSAA concentrations (r=-0.6789, p=0.001). In these experiments, recombinant goldfish C-reactive protein (rgfCRP) served as a control, and as shown in Figure 6.12A-B, rgfCRP did not affect bacterial viability.

6.3 Discussion

In human and veterinary medicine, SAA has become very sensitive marker of inflammation and/or infection status. For example, in horses SAA is used to diagnose and track prognosis of viral infections [4]. The diagnostic value of SAA in humans has been more extensively researched. It is used as a survival index in various oncological disorders [412] as well as a marker in inflammatory diseases, infection and graft rejection [413].

SAA has been implicated in numerous biological functions, however, there is contradicting evidence on the different functions of SAA. Preparations of SAA from humans are heterogeneous, and isoforms which are lipid bound are difficult to purify [414]. Consequently, commercially available recombinant SAA preparations have primarily been used in different studies. Recombinant human SAA (rhSAA) is a chimeric

of SAA1 and SAA2 containing an Asn^{61} and Arg^{72}, whereas rhSAA1 is similar to SAA1, and contains an Asp^{61} and His^{72}. The differences in biological activity have been attributed to these to amino acid modifications. For example, rhSAA has been implicated in inflammasome activation [148] and translocation of HMGB1 [415], whereas rhSAA1 is less biologically active and does not induce translocation of HMGB1 [415]. Furthermore, isoforms of SAA vary in their preference of receptors, indicating variants may have differences in modulating immune responses and inflammation [147]. Interestingly, goldfish SAA shares both Asp^{61} with rhSAA1 and Arg^{72} with rhSAA.

As an evolutionarily conserved protein, goldfish SAA possesses characteristics of the SAA superfamily including a signal peptide, SAA family signature, calcium binding domain, fibronectin-like binding domain and predicted helices domains, in relatively similar positions. The high structural conservation and similarity between goldfish SAA and mammalian and other lower vertebrate SAAs, suggested that this could also be functionally highly conserved protein.

SAA constitutive expression is widely distributed in human tissues, but predominantly located in the epithelium, where it has been suggested to play a role in maintaining homeostatic tissue functions, and also participating in host defense responses [416]. Similar to mammals, goldfish SAA was expressed ubiquitously in all tissues examined with highest expression in the kidney, spleen and intestine. The production of SAA by hepatocytes in the liver occurs during an insult or inflammation, similar to what was observed in our previous studies of *T. carassii* infections [382]. Unlike normal goldfish tissues, estuary cod had highest expression of SAA in liver, gill and muscle [409], and rock bream displayed highest expression in normal liver tissues [410]. The differences in the expression of SAA in the liver between cod and rock bream and goldfish may be due to the fact that cod and rock bream are perciformes, and are distantly related to goldfish that are cypriniformes (Fig. 6.3).

Among goldfish immune populations, SAA mRNA levels were highest in neutrophils, and lowest in macrophages. There is limited information on expression of SAA in immune cell populations of fish. In rainbow trout cell lines, SAA was up-regulated 456 fold in an epithelial cell lines (RTL) and 533 fold in a macrophage cell line

(RTS11), after stimulation with the oomycete, *Saprolegnia parasitica* [417]. Similarly, SAA had also been detected in rainbow trout macrophages challenged with LPS [418]. The lowest constitutive expression of SAA was observed in goldfish macrophages, compared to other immune cell populations. Significantly increased mRNA levels of SAA were observed in activated goldfish primary macrophages. Interestingly, extra-hepatic production of SAA has been documented in primary cell cultures and non-hepatic cell lines, which may account for very high SAA mRNA levels in studies using *in vitro* activation regimens in cultures of primary fish cells or fish cell lines [419].

Transcription of SAA has previously been correlated to pathogen load (*A. salmonicida)* in rainbow trout [149]. In addition, our group has previously shown *T. carassii* induced persistent up-regulation of SAA mRNA expression in the kidney, liver and spleen [382]. The results of this study demonstrate that parasitemia and SAA mRNA levels were positively correlated in the kidney and the spleen but not in the liver. The presence of the flagellate parasites in specific organs has been associated with successful elimination of the parasites from the host. For example, in *Trypanosoma cruzi* infected mice, elimination of parasites from the liver was related to the presence of low number of parasites in this tissue [420]. Since *T. carassii* has been shown to aggregate in internal organs such as kidney and spleen, it is perhaps not surprising to observe a positive correlation between parasite load and SAA mRNA levels [288].

IL-1β1 mRNA levels significantly increased in the liver and spleen of goldfish infected with *T. carassii* [303]. IL-1β has been implicated in the development of a Th17 response during the course of infection of carp with *T. carassii* [124]. SAA was also shown to stimulate the production of IL-23, another cytokine involved the Th17 response [124]. In a mouse model, SAA has also been suggested to contribute to Th17 allergic asthma [421]. These results suggest that high SAA mRNA levels seen during the course of *T. carassii* infection [382] may contribute to a Th17-like response resulting in a strong adaptive immune response that eventually results in elimination of the parasite from the host.

Recombinant human SAA has been documented to have pleotropic effects *in vitro*, inducing the production of both pro-inflammatory and anti-inflammatory cytokines [129].

In goldfish monocytes, similar to mammals, the expressions of pro-inflammatory cytokines TNFα1, and TNFα2, and anti-inflammatory cytokine IL-10 were significantly up-regulated at 3 hours post treatment. Human monocytes stimulated with rhSAA exhibited a similar pattern of TNFα and IL-10 gene expression [128]. The researchers [128] suggested that after induction of pro-inflammatory cytokines, SAA accumulation leads to an induction of IL-10, which in turn regulated the return to homeostasis.

Previous studies have shown recombinant human SAA can induce cell migration in human monocytes, polymorphonuclear cells (PMN) [141], and mast cells [422]. In addition, subcutaneous injection of mice with rhSAA was reported to induce neutrophilia [142]. To functionally characterize goldfish SAA, I assessed whether rgSAA can induce chemotaxis in goldfish immune populations. Chemotaxis was observed at 1 and 10 μg/mL rgSAA for both neutrophils and macrophages. Contrary to studies using mammals [141], rgSAA was not chemotactic for monocytes at any of the concentrations tested. It should be noted that a recent report demonstrated that chemotaxis of monocytes was induced indirectly, through SAA stimulation of chemokine production [423].

Macrophages activated with 5 μg/mL of rgSAA exhibited increased mRNA levels of SAA at 3, 6, and 12 hours post treatment. Secretion of SAA by macrophages has been previously documented [419], suggesting that SAA may participate in local inflammation. In human macrophages, rhSAA has been implicated in activation of the NLRP3 inflammasome and found to induce the release of mature IL-1β [144,148]. In this study, goldfish IL-1β1 gene expression was up-regulated as early as 1 hour post treatment with rgSAA, however, no changes in the expression of the gene encoding the goldfish NLRP3 was observed.

It has been reported that SAA can mediate the antimicrobial response of mouse macrophages (nitric oxide response) by binding surface toll-like receptor 4 (TLR4) [144]. In a study assessing *Acinetobacter baumanii* infections, exogenous administration of rhSAA lead to an attenuated local inflammatory response and increased susceptibility of mice to infection [424]. The rhSAA induced M2 phenotype in macrophages, suggesting SAA may be involved in resolution of inflammation [425]. Since *T. carassii* does not induce robust responses in macrophages and monocytes, I examined whether rgSAA had

the capacity to modulate the response of monocytes to heat-killed (HK) *A. salmonicida.* The results indicated that treatment of monocytes *in vitro* with rgSAA did not affect their ROI production. In contrast, treatment with rgSAA down-regulated nitric oxide response of macrophages activated by HK- *A. salmonicida*, suggesting that SAA has the capacity to dampen select antimicrobial response of phagocytes.

The rhSAA has been shown to bind Gram-negative bacteria such as *E.coli* via the outer membrane protein A (OmpaA) [426], and act as an opsonin to enhance phagocytosis of bacteria by mammalian neutrophils and macrophages [411]. In contrast, the phagocytosis of rgSAA-opsonized *E. coli* by goldfish macrophages and neutrophils of decreased significantly (Table 6.3), and when the phagocytes were pre-treated with rgSAA their phagocytic capacity was similar to non-treated cells (Table 6.4). I reasoned that the apparent differences in the phagocytic response in the presence of recombinant SAA may be masked by the direct bacteriolytic effects of rgSAA It is well established that amyloid peptides cause cellular toxicity in different diseases such as Alzheimer's (neurons) and type II diabetes (β-pancreatic cells) because they have similar properties to antimicrobial peptides (AMPs) [427]. The rhSAA was shown to have the capacity to form channels in lipid bilayers, and this property may also contribute to its bacteriolytic effect [428]. Moreover, SAA has been compared to the AMP, human cathelicidin LL-37 [429], whose heparin-binding motifs contribute to its antimicrobial properties [430]. For example, intestinal SAA reduced bacterial growth in mouse colitis, suggesting a role in intestinal immune homeostasis [431]. SAA was also shown to inhibit formation of biofilms in urinary tract infections in a mouse model [432]. Like the mammalian counterparts, goldfish SAA contains glycosaminoglycan (GAGs) on its C-terminus, defined by basic amino acid clusters present in heparin/heparan sulfate (HS) binding domains. Therefore, I examined whether rgSAA had antibacterial capabilities. The results showed that the addition of increasing concentrations of rgSAA to bacterial cultures caused a significant (30%) reduction of *E. coli* CFUs, suggesting that bacteriolytic effect of SAA may be highly conserved through evolution.

Although SAA was shown to be significantly up-regulated in goldfish in responses to infection, suggesting a role in host defense, the assessment of rgSAA effects

on pro- and anti- inflammatory cytokine gene expression in immune cells suggests that the central role of SAA may be immunomodulatory playing a significant role in maintenance of homeostasis. This does not preclude a direct role for SAA in host defense, given that the molecule participates in the regulation of antimicrobial responses of phagocytes and has a significant bactericidal effect *in vitro*.

Figure 6.1 Goldfish SAA full-length cDNA showing ORF, untranslated regions (UTR) and various domains. At the 5' and 3' ends, the UTR is shown in upper case letters. The open reading frame (ORF) is in lower case letters, start and stop codons are double underlined. The SAA protein domain is marked with dark grey. The signal peptide is underlined.

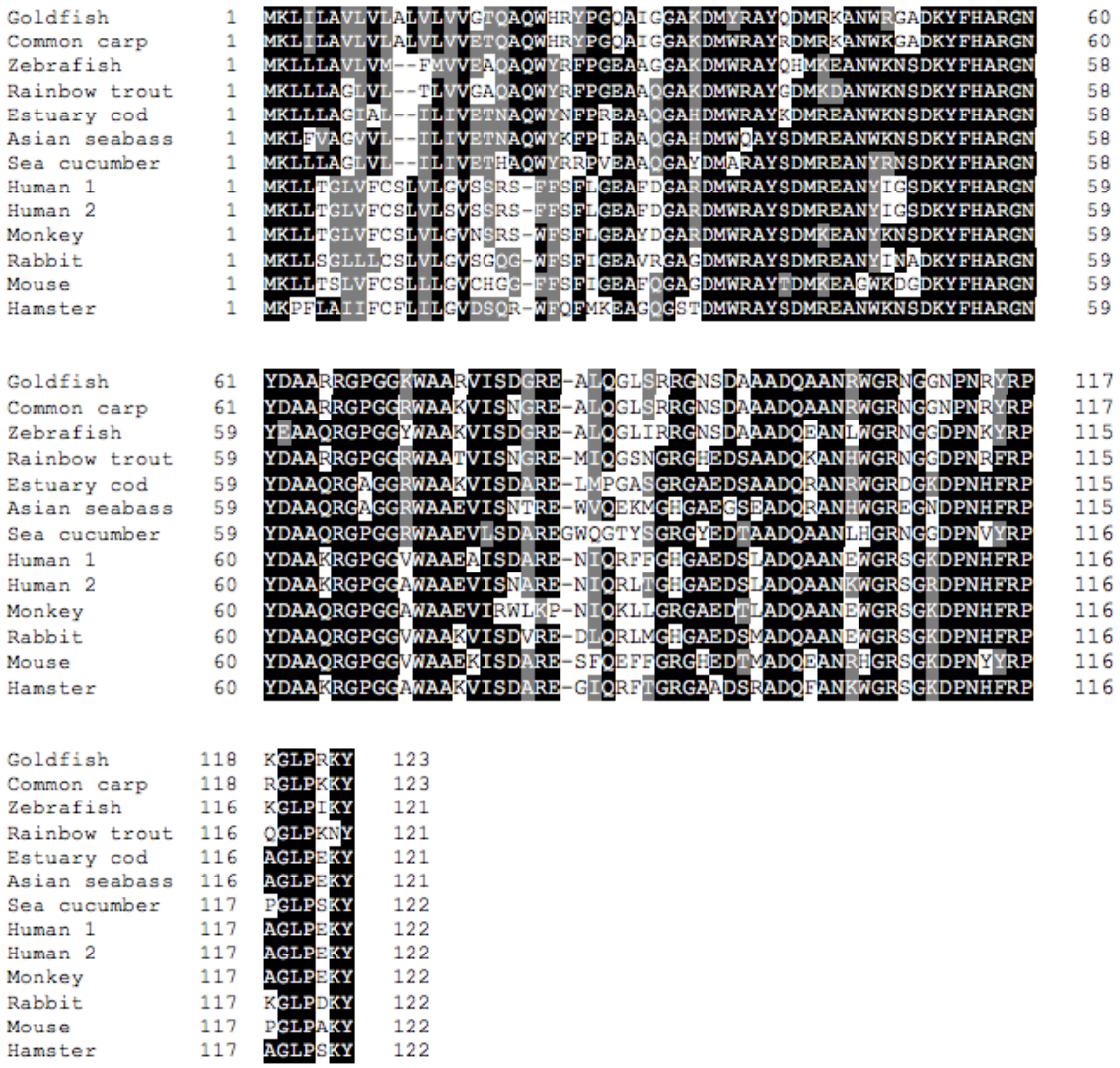

Figure 6.2 Protein alignment of goldfish SAA with other known SAA sequences. Black shaded sequence indicates residues that are fully conserved, gray shaded sequences indicate conserved amino acids substitutions, light gray indicate sequence with semi-conserved amino acid substitutions and dashes indicate gaps. GenBank accession numbers are as follows: Common carp (BAA36700.1), Estuary cod (AFQ00088.1), Asian seabass (ADE05545.1), sea cucumber (AAG24633.1), rainbow trout (CAM12348.1), zebrafish (NP_001005599.1), monkey (EHH23058.1), human 1 (AAA64799.1), human 2 (AAH20795.1), rabbit (CAA34451.1), mouse (AAA40085.), and hamster (AAA37098.1)

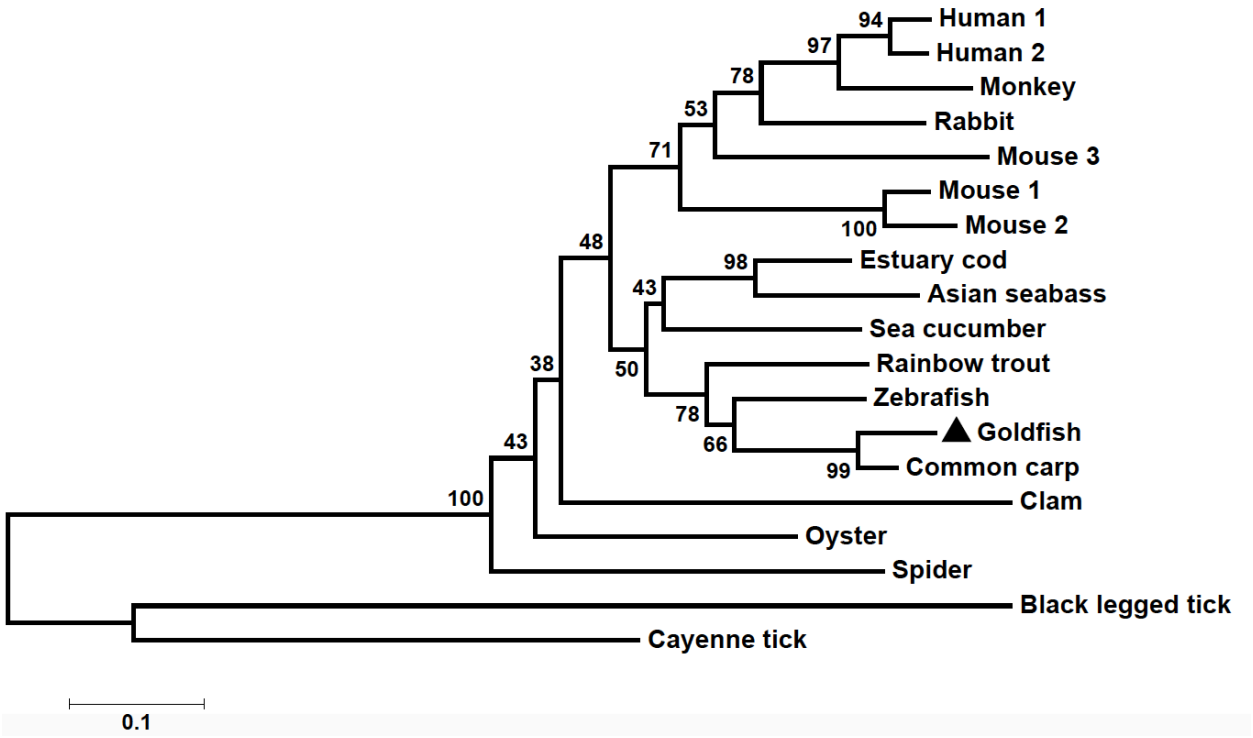

Figure 6.3 Phylogenetic analysis of goldfish SAA against SAA of other known isoforms in vertebrates. Amino acid sequences were aligned by using CLUSTAL-Omega program and an unrooted phylogenetic tree was constructed using the neighbor-joining method of the MEGA 5 software. The tree was bootstrapped 10,000 times. GenBank accession numbers are as follows: Common carp (BAA36700.1), Estuary cod (AFQ00088.1), Asian seabass (ADE05545.1), sea cucumber (AAG24633.1), rainbow trout (CAM12348.1), zebrafish (NP_001005599.1), monkey (EHH23058.1), human 1 (AAA64799.1), human 2 (AAH20795.1), rabbit (CAA34451.1), mouse 1 (AAH87933.1), mouse 2 (AAB37250.1), mouse 3 (AAH55885.1), clam (AJF23095.1), oyster (AHY23235.1), spider (AGR53514.1), black legged tick (EEC20416.1), and cayenne tick (JAC23922.1).

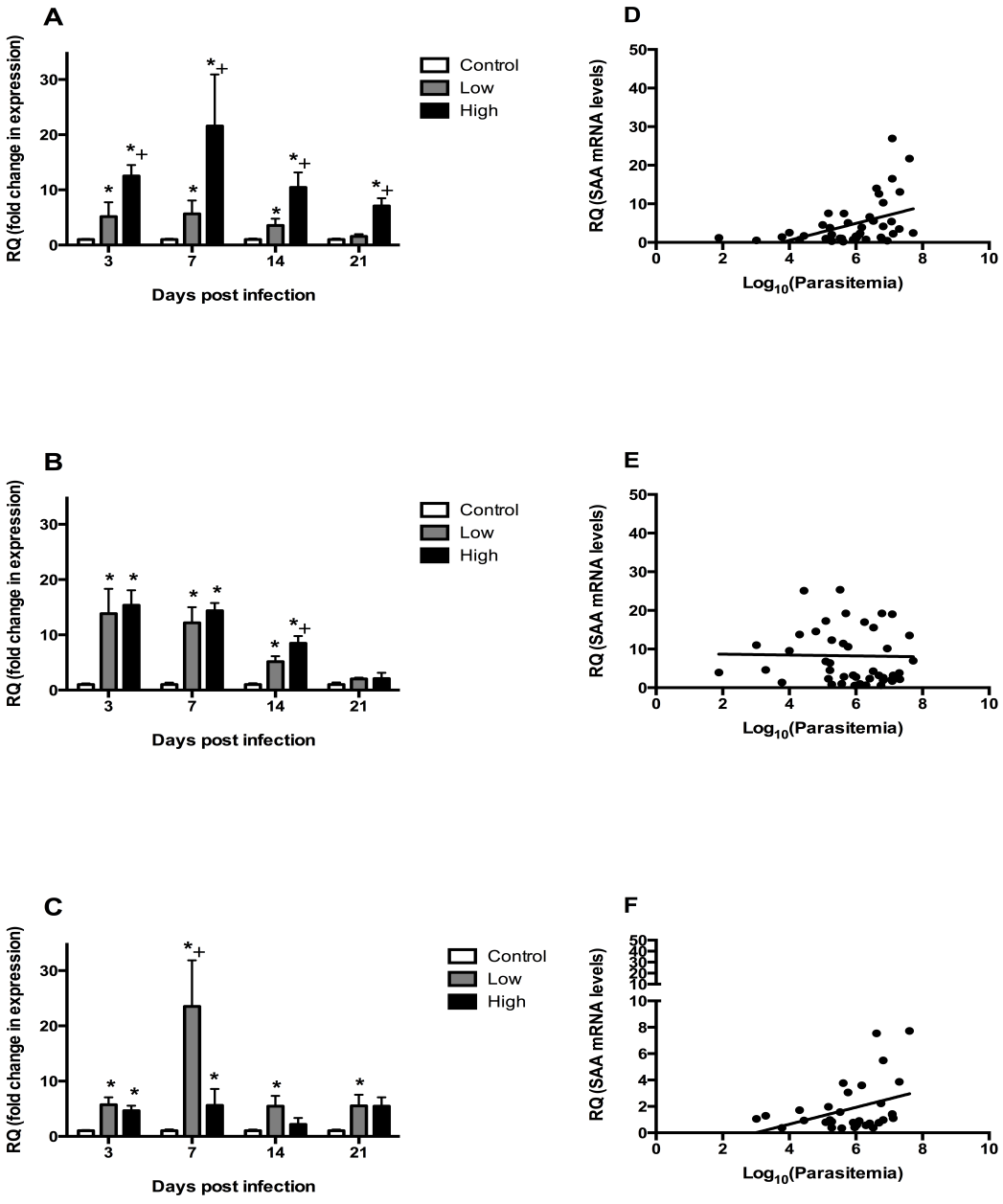

Figure 6.4 Quantitative expression analyses and correlation of goldfish SAA expression in goldfish infected with two doses of *T. carassii* and non-infected goldfish (A/D) kidney **(B/E)** liver **(C/F)** spleen levels. Expression was relative to endogenous control gene, elongation factor 1 alpha (EF-1α) and normalized against those observed in non-infected control. Data are represented as mean ± SEM of 6 fish ($P < 0.05$). Asterisks (*) denote statistical significance from non-infected control fish. Statistical analysis was performed using two-way ANOVA followed by Dunnett's post hoc test. Asterisks (*) denote statistical significance from non-infected control fish, and plus sign (+) indicates statistical significance between treatment groups ($P < 0.05$).

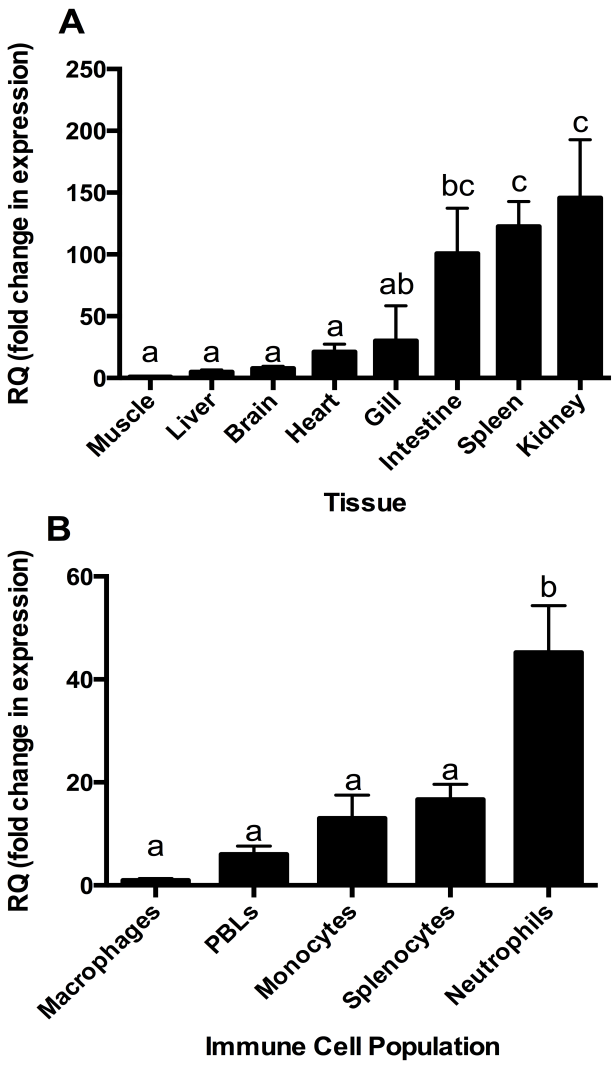

Figure 6.5 Quantitative constitutive expression analyses of goldfish SAA expression in goldfish tissues and immune cell populations obtained from normal fish. Goldfish SAA expression in goldfish tissue and immune cell populations is relative to endogenous control gene, elongation factor 1 alpha (EF-1α). The mean Ct value for EF-1α was in the range of 21.54 - 21.8 and 19.34-19.76 for all immune cell populations and tissues, respectively. **(A)** Goldfish SAA tissue analysis. Analysis of the relative expression data are pooled from 5 individual, performed in triplicate, and normalized against muscle. **(B)** Goldfish SAA expression in non-stimulated goldfish immune cell populations. The cells examined were: monocytes, macrophages, peripheral blood leukocytes (PBL), kidney-derived neutrophils and splenocytes. Analysis of the relative expression data are pooled from 5 individual, performed in triplicate, and normalized against FACS-sorted macrophages. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Different letters above each bar denote statistically different ($P < 0.05$), and the same letter indicates no statistical differences between groups.

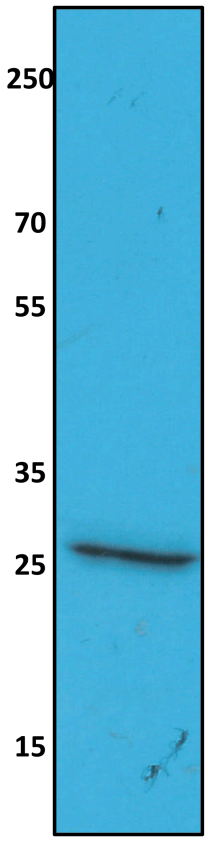

Figure 6.6 Western blot analysis of recombinant goldfish SAA. Recombinant goldfish SAA was expressed in a prokaryotic system and purified as described. rgSAA is recognized by mouse anti-histidine monoclonal antibody (1:5000) and visualized using chromogenic ECL development kit.

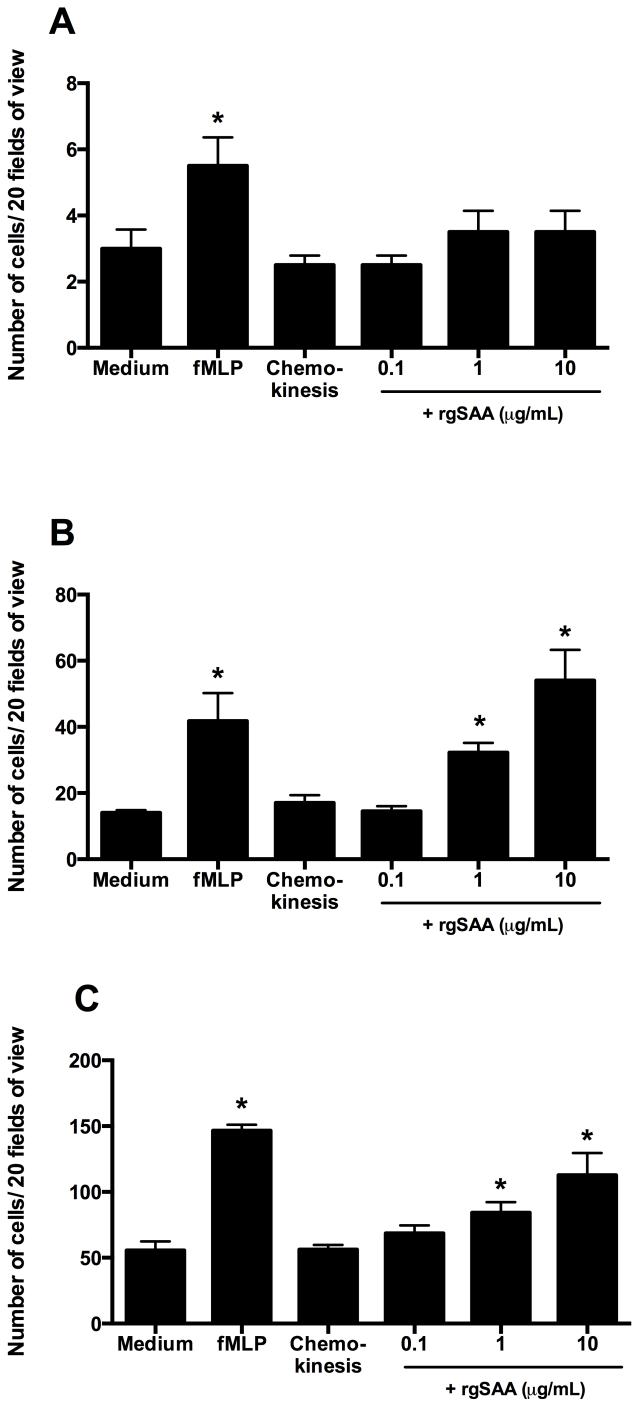

Figure 6.7 Chemotactic activity of goldfish immune cell populations induced by recombinant goldfish SAA. Chemotaxis was measured by counting 20 random fields of view under oil immersion (100X). Chemotactic activity was determined as the mean ± SEM number for **(A)** monocytes **(B)** mature macrophages and **(C)** kidney-derived neutrophils from 4 individual fish. Statistical analysis was performed using a one-way ANOVA followed by a Dunnet's post hoc test. Asterisks (*) denote significant differences compared to medium control ($P < 0.05$).

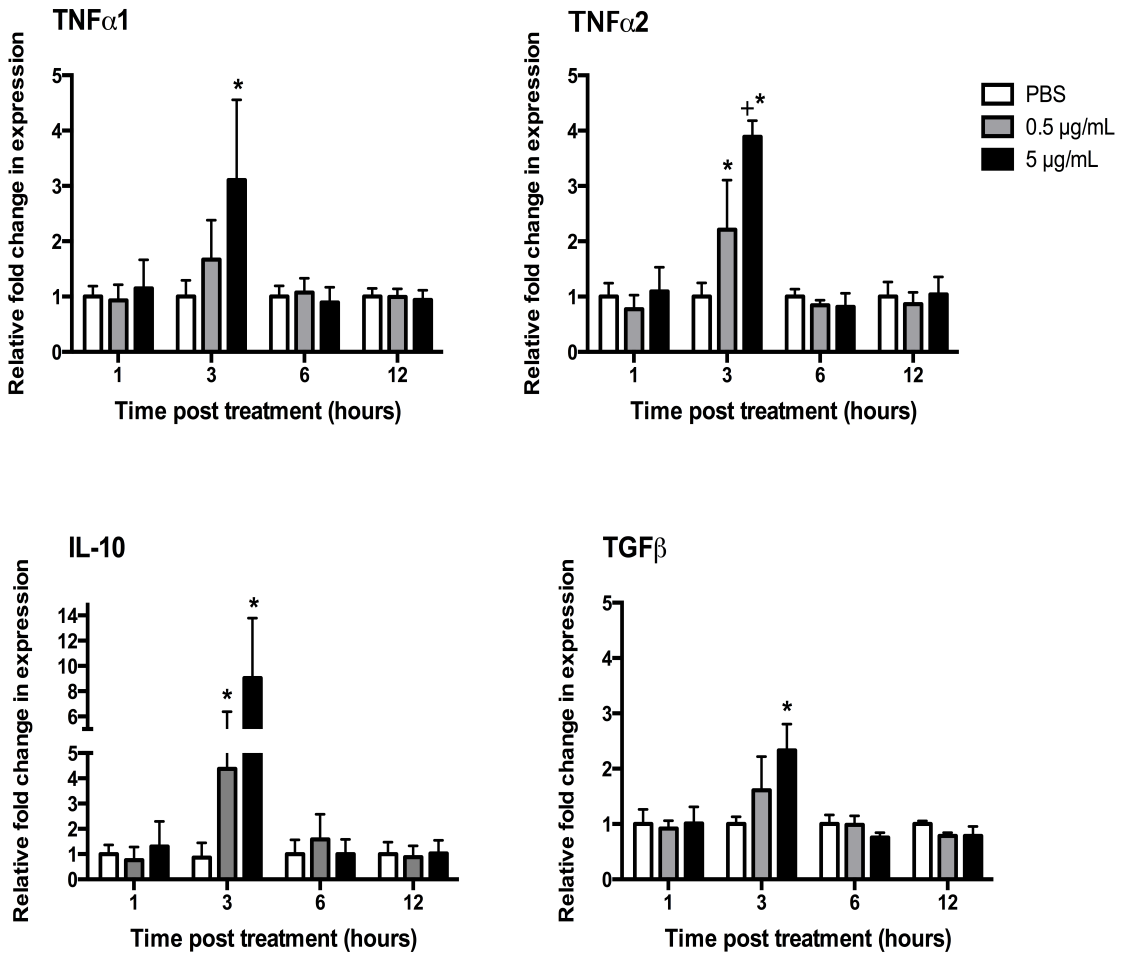

Figure 6.8 Quantitative expression analysis of goldfish pro-inflammatory and anti-inflammatory cytokines in SAA-treated monocytes. Monocytes were treated with 0.5 µg/mL, 5 µg/mL rgSAA or PBS control for 1, 3, 6 and 12 hours. The expression of goldfish cytokines was examined relative to endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data was normalized against those observed for each gene for each time point. The relative expression for each cytokine was presented as the mean ± SEM of 4 separate cultures established from individual fish (n=4). Asterisk (*) indicates statistical significance ($P < 0.05$) from PBS-treatment control, and plus sign (+) indicates statistical significance between treatment groups.

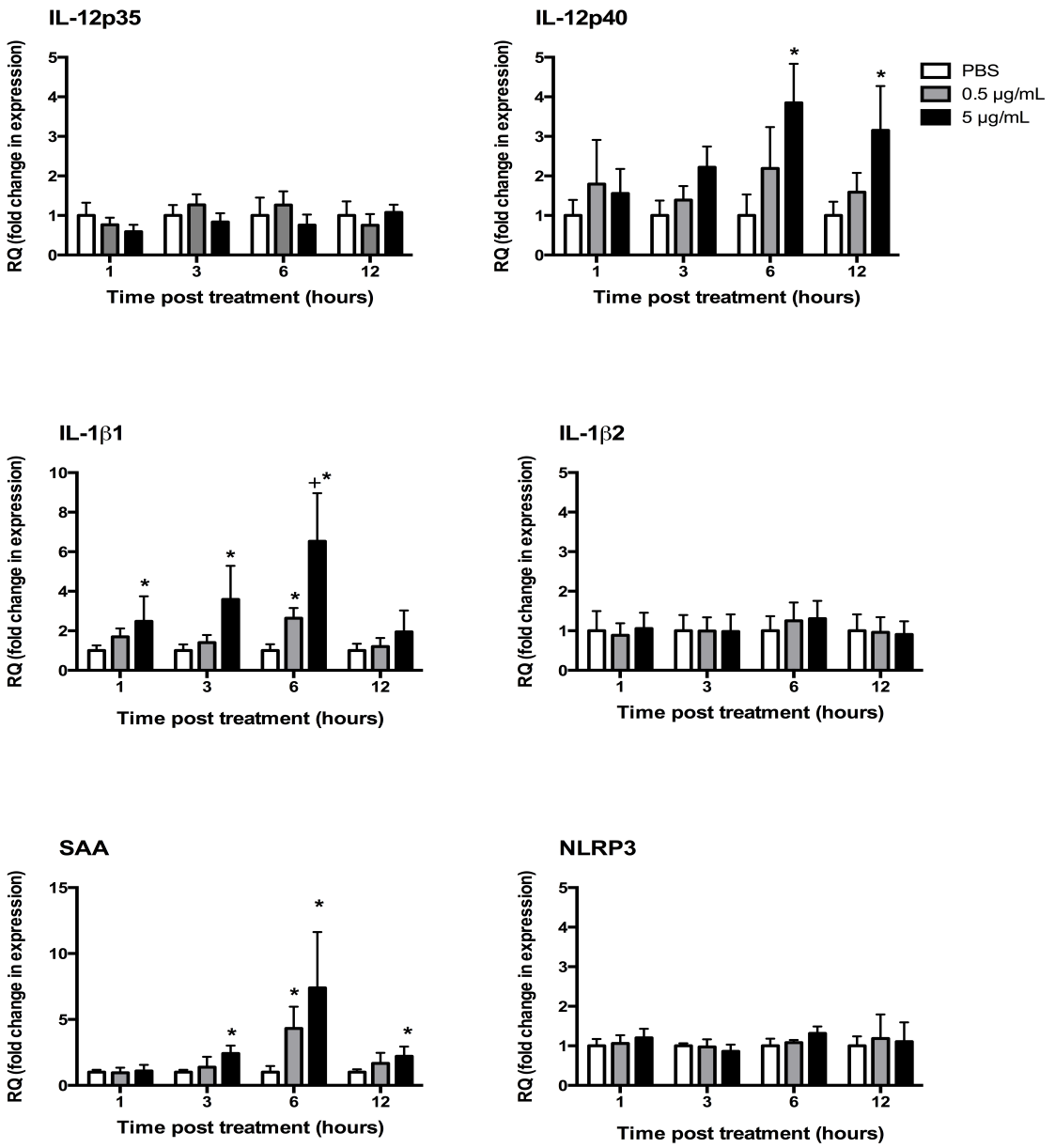

Figure 6.9 Quantitative expression analysis of select goldfish cytokines in SAA-treated macrophages. Macrophages were treated with 0.5 µg/mL or 5 µg/mL rgSAA or PBS control for 1, 3, 6 and 12 hours. The expression of goldfish cytokines was examined relative to endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data was normalized against those observed for each gene for each time point. The relative expression for each cytokine was presented as the mean ± SEM of 4 separate cultures established from individual fish (n=4). Asterisk (*) indicates statistical significance ($P < 0.05$) from PBS-treatment control, and plus sign (+) indicates statistical significance between treatment groups.

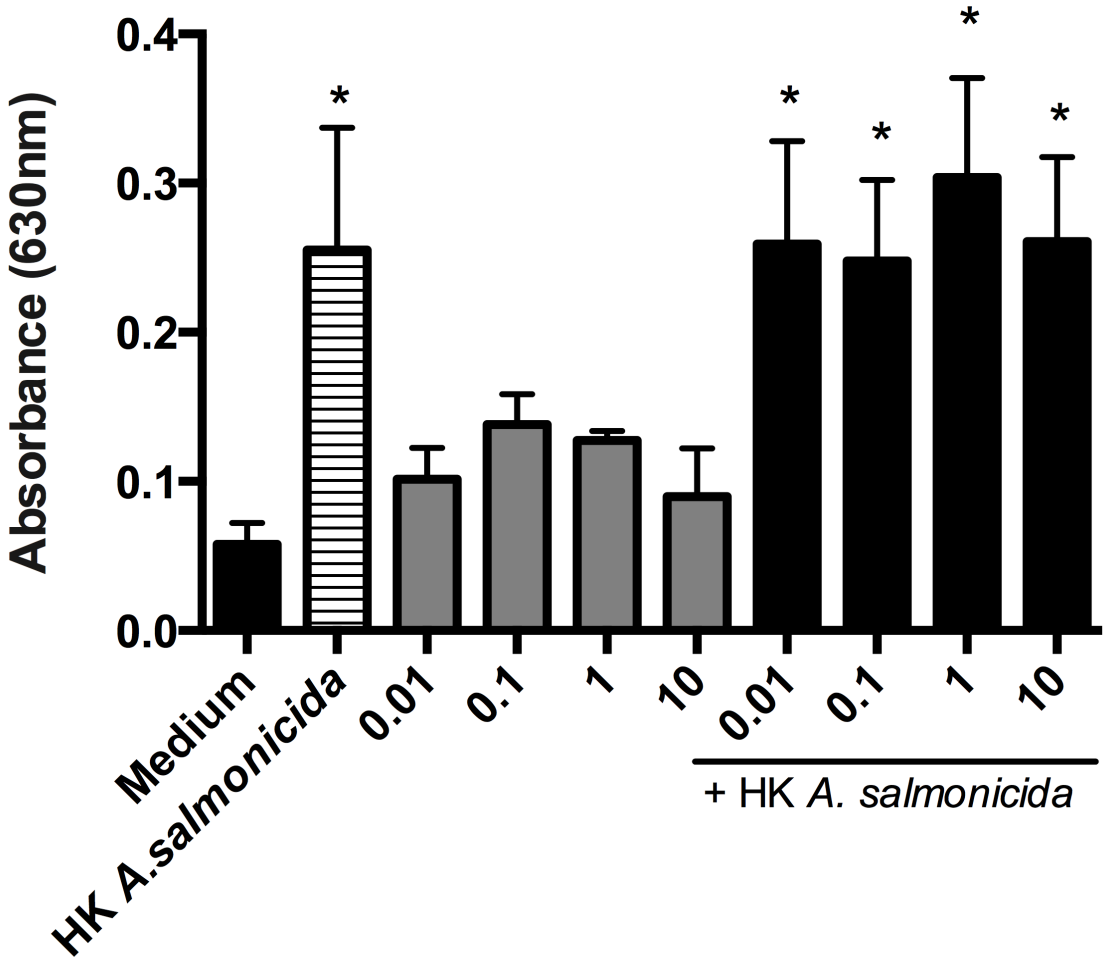

Figure 6.10 Recombinant goldfish SAA does not affect the production of reactive oxygen intermediates in monocytes activated by HK-*A. salmonicida.* Monocyte cultures were pre-treated with varying concentrations of rgSAA or medium for 3 hours and activated using heat-killed *A. salmonicida.* ROI assays were incubated for 18 hours at 20°C. Production of reactive oxygen species was determined using the NBT reduction assay. The absorbance values of medium-treated controls (no PMA) were subtracted from treatment values to factor in background NBT. Data are presented as mean ± SEM of ROI from 5 individual fish (n=5). Statistical analysis was performed using a one-way ANOVA and a Tukey's post hoc test. Asterisks (*) denote significant differences compared to negative control (PMA only) ($P < 0.05$).

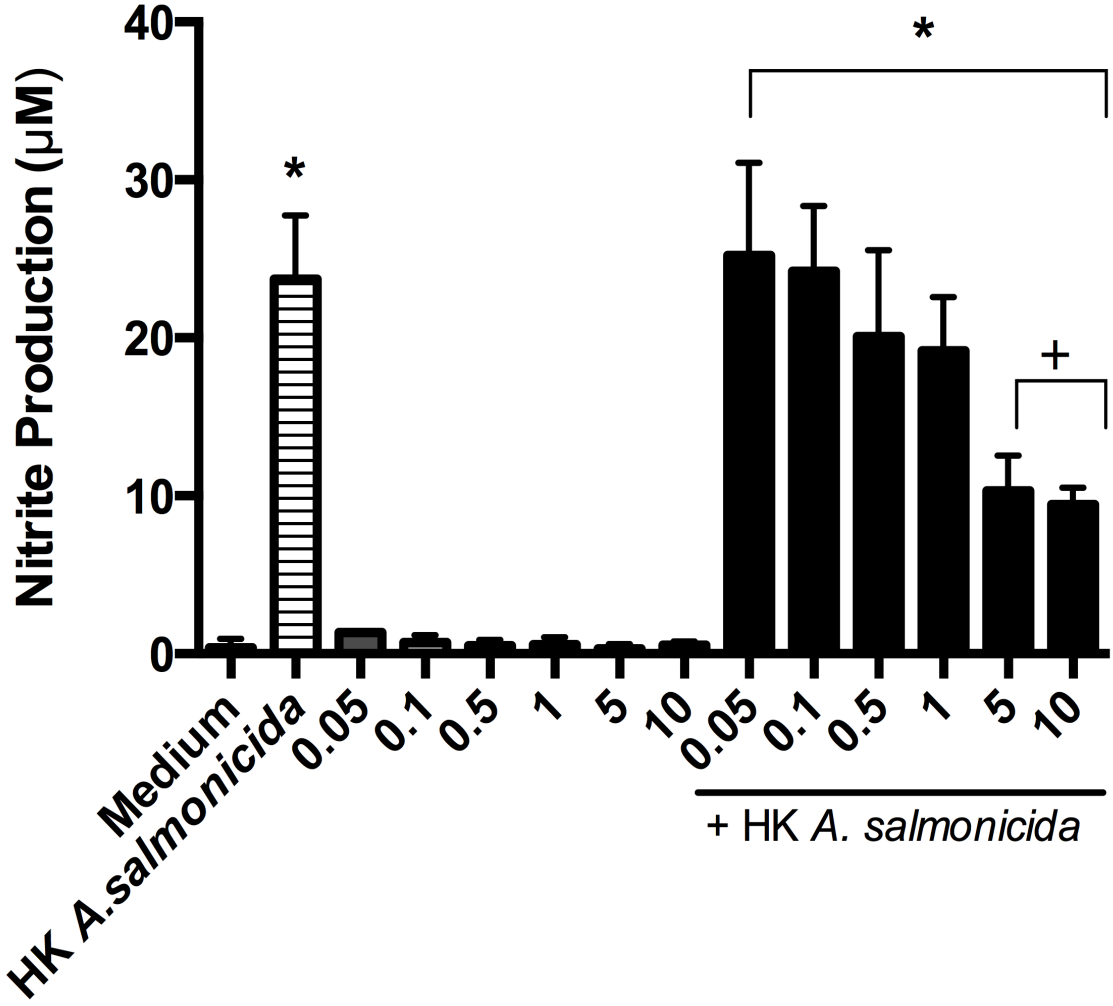

Figure 6.11 Recombinant goldfish SAA suppressed nitrite production in macrophages activated with HK-*A. salmonicida.* NO production by goldfish mature macrophages was determined using the Griess reaction where production of nitrite was determined using a nitrite standard curve. The mean ± SEM nitrite production was determined from culture established from six individual fish (n=6). Statistical analysis was performed using a one-way ANOVA and a Tukey's post hoc test. Asterisks (*) denote significant differences compared to medium control and plus sign (+) denotes statistical difference from positive control ($P < 0.05$).

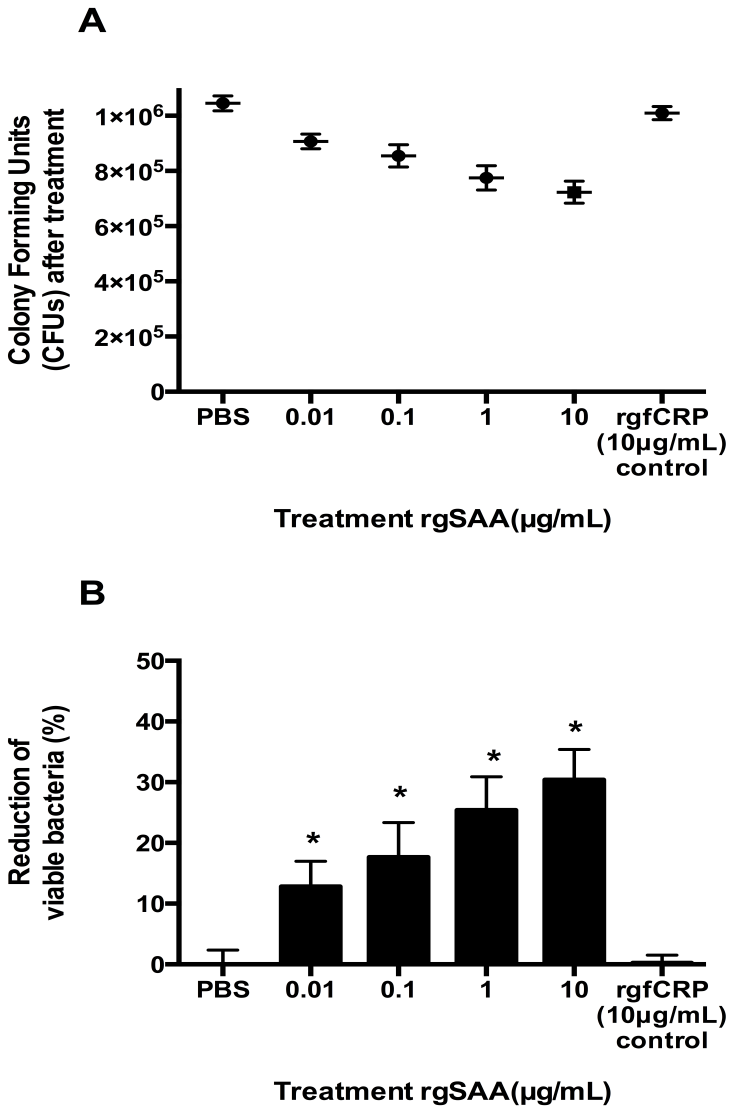

Figure 6.12 rgSAA affected viability of *E.coli*-DH5α. (A) Number of viable bacteria after addition of different amounts of rgSAA. (B) Percent reduction in viable bacteria in cultures. Bacterial viability was determined by enumerating colony forming units (CFU). Each point represents the average of 5 independent experiments (*n*=5) performed in duplicate. The percent reduction of viable bacteria was calculated by normalizing each rgSAA treated group against that of PBS treated *E.coli*-DH5α.

Table 6.1 Percentage identity and similarity between goldfish SAA with other known SAA species.

Species	% Identity	% Similarity
Common carp	92.74	97.58
Zebrafish	76.61	84.67
Rainbow Trout	70.96	81.45
Estuary cod	63.7	75.8
Asian seabass	58.06	72.58
Sea cucumber	66.93	73.38
Human 1	28.62	71.77
Human 2	28.62	71.77
Monkey	55.64	70.16
Rabbit	62.9	73.38
Mouse	55.64	66.12

Table 6.2 Fold index summary of goldfish SAA with other known SAA species

	Number of disordered regions	Longest disordered regions	Number of disordered residues	Disordered residues as a percentage
Goldfish	1	91	91	73.98
Common carp	1	92	92	74.79
Zebrafish	2	44	88	72.72
Rainbow Trout	1	90	90	74.38
Sea Cucumber	1	90	90	73.77
Monkey	1	82	82	67.21
Mouse	1	82	82	67.21
Rabbit	2	52	60	49.18
Human1	3	52	78	63.93
Human 2	3	52	79	64.75

Table 6.3 Recombinant goldfish SAA does not act as an opsonin to enhance phagocytosis of *E.coli* in goldfish neutrophils or macrophages[1]

	Percentage Phagocytosis (Mean ± SEM)	
Sample	Neutrophils	Macrophages
E.coli DH5α-GFP	20.94 ± 1.69	29.35 ± 9.00
E.coli DH5α-GFP + 0.01μg/mL rgSAA	10.36 ± 0.68*	11.93 ± 0.83
E.coli DH5α-GFP + 0.1μg/mL rgSAA	6.07 ± 0.44*	12.55 ± 0.15
E.coli DH5α-GFP + 1μg/mL rgSAA	9.06 ± 0.81*	12.30 ± 3.03
E.coli DH5α-GFP + 10 μg/mL rgSAA	8.42 ± 1.67*	12.78 ± 1.62

[1]*E.coli* DH5α-GFP opsonized for 30 minutes with varying concentration of recombinant goldfish SAA. Samples were then incubated with goldfish neutrophils (n=3) for 30 minutes or with macrophages (n=2) for 1 hour, after which cells were fixed and phagocytosis was analyzed using Cell Lab Quanta SC flow cytometer. Statistical analysis was performed using a one-way ANOVA and a Dunnett's post hoc test. Asterisks (*) denote significant differences compared to PBS control ($P < 0.05$).

Table 6.4 Priming with recombinant goldfish SAA does not enhance phagocytosis of *E. coli* DH5α-GFP by kidney-derived neutrophils or mature macrophages[1]

	Percentage Phagocytosis (Mean ± SEM)	
Sample	Neutrophils	Macrophages
E.coli DH5α-GFP	57.1 ± 2.41	36.6 ± 4.09
E.coli DH5α-GFP + 0.01μg/mL rgSAA	47.6 ± 0.96	29.3 ± 13.14
E.coli DH5α-GFP + 0.1μg/mL rgSAA	53.0 ± 4.48	33.8 ± 5.14
E.coli DH5α-GFP + 1μg/mL rgSAA	59.6 ± 2.00	37.1 ± 10.36
E.coli DH5α-GFP + 10 μg/mL rgSAA	57.4 ± 2.27	37.3 ± 6.3

[1]Kidney-derived neutrophils (n=3) or mature macrophages (n=3) were primed with varying concentrations of rgSAA. *E.coli* DH5α-GFP was added to cells and incubated for an additional 1 hour for neutrophils or 2 hours for macrophages. Cells were fixed and phagocytosis was analyzed using Cell Lab Quanta SC flow cytometer. Statistical analysis was performed using a one-way ANOVA and a Dunnett's post hoc test. Asterisks (*) denote significant differences compared to PBS control ($P < 0.05$).

CHAPTER 7

GENERAL DISCUSSION

7.0 Introduction

The main objective of my thesis was to (1) examine the change in genes encoding acute phase proteins in goldfish infected with *T. carassii* during the acute and chronic phases of infection and (2) based on the expression analysis, select prominent acute phase proteins for further molecular and functional characterization. Under normal conditions, *T. carassii* is not pathogenic, suggesting fish are able to control the progression of infection, and eventually eliminate the parasites [12]. However, in aquaculture setting, where fish are in high-density conditions where metabolites accumulate and water quality declines, the prevalence of infection is higher resulting in increased morbidity and morality. My thesis research enhanced our understanding of how fish modulate innate immune events during the course of a parasitic infection. The results of my thesis research represent the first comprehensive analysis of the acute phase response during the course of parasitic infections of bony fish, and also first comprehensive analysis of two major acute phase proteins, C-reactive protein (CRP) and Serum Amyloid A (SAA).

7.1 Overview and discussion of findings

In this thesis, I reported on the tissue expression of genes that encode acute phase proteins (APPs) during the course of infection of goldfish with *T. carassii.* Given the documented variability in APP gene expression induced by different inflammatory stimuli, it was important to assess the expression of a number of APPs [3]. During an inflammatory insult, the concentrations and kinetics of APPs are related to severity of damage and the time course of inflammatory events [433]. Although APPs are typically associated with an acute response, they are not a feature of only acute infections. When a specific stimulus persists, a sustained chronic acute phase response have been described [351]. Since the course of infection with *T. carassii* in goldfish can last weeks, before the parasite is eliminated from the blood, I decided to assess the acute phase response (APR) during both acute and chronic phases of the infection.

During the acute course of *T. carassii* infection, I observed significant changes of genes encoding APPs in the liver. This was not unexpected since the liver is the main site of APP synthesis, although extra-hepatic synthesis of APP has also been documented for select APPs [89]. The observed up-regulation of iron regulatory proteins suggests that the host may be using an 'iron withholding strategy' to limit iron availability and prevent pathogen proliferation. Cp is necessary for the oxidation of ferrous iron to ferric iron, such that Tf bound to ferric iron can be transported to target cells or organs [158]. Increased Tf levels can generate environments with low iron availability [158,161], which may influence *T. carassii* growth and development [327].

During the chronic phase of infection, the mRNA levels of genes encoding APPs in the liver were similar between infected and non-infected goldfish, suggesting that APPs do not play a major role during the elimination phase of the infection. It is well established that anti-parasite antibodies are responsible for clearance of *T. carassii* and for resistance of fish to re-infection [277,281,283]. Antibody titres in goldfish [283] and salmonids peak 2-3 weeks after infection, however this response can take longer at lower temperatures [434]. The decline in mRNA levels of genes that encode APPs, compared to the mRNA levels of APPs during the acute phase of the infection, coincide with the rise of anti-parasite antibody levels [281,283], suggesting that APR in the liver is down-regulated once the host mounts an effective adaptive immune response.

In contrast to my observations in the liver, I observed a down-regulation of iron regulatory proteins in the kidney and the spleen during the acute course of *T. carassii* infection. Induction of APPs in the liver may play a role in immunomodulation, however changes in APPs in other tissues such as the spleen and kidney may be related to homeostatic functions. Enhanced phagocytosis of erythrocytes has been documented in other trypanosome infections [333], and may be a strategy used by the parasites to balance iron availability, since iron withholding may be employed by the host. Down-regulation of Cp, Tf and Hx were observed during the acute phase of infection in the kidney and spleen suggesting a shift from iron storage to iron availability. Contrary to the acute infection, in the chronic phase of infection, these iron regulatory proteins were

significantly up-regulated, suggesting an overcompensation effect may be occurring to balance iron levels.

The initial expression analysis of APPs indicated that both SAA and CRP were highly expressed during *T. carassii* infection. Consequently, I choose to further characterize these two APPs at the molecular and functional levels. In Chapter 5, I describe studies whose main aim was the characterization of CRP.

CRP is a systematic marker of inflammation in mammals, and has also been shown to be an important mediator of inflammation [110]. For a protein that has been studied extensively for 85 years, much remains to be elucidated regarding its role in inflammatory conditions. In contrast to results of some early studies using mammals [97,110], I report that recombinant goldfish CRP does not have pro-inflammatory properties such as activation of antimicrobial effector functions in goldfish monocytes and macrophages. It should be noted that recent studies using mammals reported that the initial results attributing pro-inflammatory properties to CRP may actually be due to either lipopolysaccharide and/or sodium azide contamination, commonly found in commercial preparations [435,436]. To avoid artifacts caused by commercial preparations, *in vivo* animals models have been widely used despite the numerous caveats associated with certain models. For example, transgenic mice overexpressing human CRP are commonly used to study cardiovascular disease. However, human CRP is a foreign protein in mice, and is controlled by testosterone, restricting meaningful analysis of data [437].

Furthermore, recombinant gfCRP was not able to act as an opsonin to enhance phagocytosis of *T. carassii* by macrophages. It is well established that *T. carassii* possesses surface molecules that are used by the parasite to evade host immune responses [9,312]. For example, *T. carassii* gp63 abrogated fish macrophage antimicrobial responses [312], and *T. carassii* calreticulin (CRT) has been shown to bind C1q preventing downstream effector functions [9]. Recent work on human CRP has elucidated differences in inflammatory profiles of different conformations of CRP. The native pentameric CRP (pCRP), which was once believed to be the important in inflammation, has been recently replaced with the monomeric or modified CRP (mCRP).

Both mCRP and pCRP can bind C1q to activate classical complement pathway [399]. However mCRP expresses neoantigens distinct from the pCRP, causing it to selectively bind immune complexes, and thus be much more efficient in activation of the classical complement pathway [438]. In human medicine, there are techniques to distinguish between different isoforms of CRP, which are lacking for fish [439]. Therefore, whether recombinant goldfish CRP is one subunit of pCRP or is present as mCRP in goldfish is uncertain and requires future investigation once the techniques become readily available. Interestingly, my results showed that the recombinant goldfish CRP enhanced complement-mediated lysis of *T. carassii in vitro*, as indicated by small but significant reductions of viable parasites in cultures. Given that *T. carassii* persisted and proliferated in the host despite elevated CRP, suggests that this APP may not play a central role in preventing establishment of the parasite during the acute phase of the infection.

The results of molecular and functional characterization of goldfish SAA have been presented in Chapter 6 of this thesis. Since early 1970's, SAA has been extensively studied in various disease states such as cardiovascular diseases and oncological states in humans. Similar to CRP, differences in functional effects of commercial preparations of recombinant SAA have been reported in the literature [440]. Differences in amino acid modifications of recombinant proteins have been shown to contribute to its biological activity [147]. Moreover, the immunomodulatory effects attributed to SAA appear to differ systemically and at inflammatory sites. For example, SAA is up-regulated systemically in response to infection, yet it has been shown to attenuate local inflammatory responses [424].

In silico analysis of goldfish SAA indicated that it was highly conserved, and that it shared distinctive signature motifs of the SAA superfamily. Given the highly conserved nature of SAA, I postulated that goldfish SAA would have similar functional properties to its mammalian counterpart. SAA was ubiquitously expressed in all normal goldfish tissues, and its constitutive expression was highest in neutrophils and lowest in macrophages. However, when macrophages were stimulated with rgSAA, the mRNA levels of the gene encoding SAA was significantly elevated, suggesting that during the inflammatory response, SAA may play an immunomodulatory role.

To determine whether there was a relationship between SAA mRNA levels and *T. carassii* load in goldfish, I determined parasitemia in fish in parallel to measuring SAA mRNA levels during the course of the infection. Goldfish SAA mRNA levels were positively correlated to parasitemia in the kidney and spleen, but not the liver. It has been reported that *T. carassii* aggregates in the kidney and spleen of goldfish [288], which may explain why SAA mRNA levels in these tissues were related to parasitemia.

Similar to mammalian counterparts, rgSAA displayed chemotactic activity for goldfish macrophages and neutrophils however did not induce monocyte chemotaxis. Previous research has shown recombinant human SAA to be chemotactic for a variety of immune populations, however recent research has shown that monocyte chemotactic activity is mediated by the induction of chemokines by rhSAA rather than rhSAA *per se* [423].

To assess the effects rgSAA on goldfish macrophages and monocyte antimicrobial functions, I used HK-*A. salmonicidia* to activate the cells, since research in our laboratory has shown that exposure *T. carassii* does not activate goldfish phagocytes [257]. Recombinant goldfish SAA did not induce reactive oxygen intermediate production, however, it did induce an up-regulation of pro-inflammatory (TNFα1 and TNFα2) and anti-inflammatory (IL-10) cytokines. In contrast to results of studies using mammals, where recombinant SAA was reported to induce production of nitric oxide [144], goldfish recombinant SAA suppressed the nitric oxide response of macrophages activated with HK-*A. salmonicida.* Together, these results suggest goldfish SAA may play an immunomodulatory role for maintenance of homeostasis, since its effects are both pro- and anti-inflammatory.

Interestingly, amyloid peptides (such as SAA), have similar properties to antimicrobial peptides (AMPs), and SAA has been compared to the human AMP, cathelicidin LL-37 [427,429]. To determine whether this is a conserved feature of SAA, I examined whether rgSAA directly affected bacterial viability. The results indicated that incubation of *E.* coli with rgSAA reduced *E. coli* CFUs in a dose-dependent manner.

The opsonization of Gram-negative bacteria with recombinant human SAA has been shown to enhance phagocytosis [411]. In contrast, opsonization of *E. coli* with recombinant goldfish SAA did not enhance phagocytosis of the bacteria by either macrophages or neutrophils. Since rgSAA affects the viability of bacteria directly (as discussed above), this may have influenced the phagocytosis assay readout, and hence no significant changes in the uptake of opsonized bacteria was observed following treatment of goldfish phagocytes with rgSAA. It has been reported that differences in specific receptor engagement with SAA, may contribute to diverse antimicrobial effector functions [127,140,144,411,441]. For example, human recombinant SAA has been shown to bind several receptors (e.g. TLR2, P2X7) and engagement of different receptors may induce different functional outcomes, which could affect phagocytosis. For example, recognition of SAA by P2X7 receptor causes inhibition of neutrophil apoptosis, and binding of TLR2 induces activation of NLRP3 inflammasome in phagocytes [148,421]. Similar modulation of macrophage phagocytosis may occur during uptake of rgSAA opsonized bacteria. Unfortunately, the lack of specific reagents did not allow me to fully examine the interaction between SAA, infectious agents and goldfish phagocytes.

7.2 Future directions

There are limited tests for assessing and diagnosing piscine welfare. Assessment of morphological features such as observations of external characteristics or behavioural changes have been used as indications of health [442]. However, fish infected with pathogens appear normal, therefore these approaches are limited in sensitivity and precision. The obvious use of APP and the ensuing APR would be for clinical or subclinical diagnosis. Although CRP is the prototypical determinant of inflammation, the levels of CRP can be inhibited by IFN, such as in certain viral infections or autoimmune diseases [443]. Additionally, baseline levels of CRP are dependent on polymorphisms of the gene [102]. SAA levels have been documented to increase more so then CRP, therefore SAA may be a more appropriate marker of inflammatory states [444]. Measurement of gene expression does not necessarily coincide with protein expression, and would require euthanasia of fish. Moreover, the gene expression of SAA in organs

may be influenced by pathogen load in specific tissues, as I have shown in my studies. Based on the above, I think that development of an antigen-capture ELISA (enzyme-linked immunosorbent assay) to measure SAA levels within serum of fish would be invaluable for the assessment of health of fish exposed to various stressors (biotic and abiotic). In order to develop the ELISA for the measurement both polyclonal and monoclonal anti-rgSAA need to be developed and tested for specificity. In addition, properly controlled experiments need to be conducted, to determine the diagnostic SAA levels following exposure of fish to specific biotic and abiotic stressors. In veterinary medicine, SAA levels are currently being used to differentiate between healthy cattle and those harboring infections or other inflammatory disease conditions [445].

The goldfish APPs identified and characterized in this study can also be used to monitor water quality and safety of drinking water and reuse water. For example, CRP was shown to be elevated in fish exposed to water containing non lethal doses of cadmium and mercury [116]. Furthermore, trout exposed to environmental chemicals that induce cytochrome P450, also induced the production of CRP [446]. The battery of APPs identified in this study can be employed in a similar manner, enhancing our understanding of the immunotoxicity induced by chemical pollutants present in water, such that we can better protect aquatic and public health.

One of the most sustainable methods to prevent pathogen-associated morbidity is gene selection for disease resistance. In certain instances, APPs levels have been found to be different between susceptible and resistance organisms. For example, mice displaying higher levels of haptoglobin were more resistance to *T. brucei* infections [447]. Future work on developing APPs as markers for disease resistance in aquaculture setting would undoubtedly enhance the productivity of this agricultural sector. The approaches used would include selective breeding and selection of resistant fish populations, using SAA levels as indicators of the resistance, rather than challenges with specific pathogens. This would significantly decrease the cost as well as decrease the evaluation time during breeding/selection process.

At present, one of the prominent areas of immunological investigation is the interaction between innate and adaptive immune responses. Not surprisingly, the research

in this area is significantly more advanced in mammals compared to teleosts [98,448–453]. As demonstrated in this thesis, acute phase proteins are important "bridge" molecules between innate and adaptive immunity. For example, CRP functions as a first line of defense by binding various pathogens, engaging with numerous receptors and promoting clearance of debris [98]. The ability of CRP to activate the complement system places it at the interface between innate and adaptive immunity, since the complement system orients adaptive immune responses as illustrated by interaction with TLRs [454], and regulation of B [455] and T cell immunity [456]. Similarly, it has been suggested that SAA aids in the development of adaptive immunity [457,458] as demonstrated by its immunmodulatory properties to activate the inflammasome [421] and stimulate production of cytokines such as TNF and IL-10 [128,129] which can provide an effector pathway to regulate adaptive immunity [459]. B and T-lymphocytes are the main components of the adaptive immune system [460], therefore development of tools to distinctively distinguish between lymphocyte subsets and techniques to measure lymphocyte effector functions such as immunological-based assays or microarrays/Q-PCR to asses gene expression of genes related to B and T-lymphocytes (i.e. surface markers) would be required to functionally determine the role of APPs at the interface between innate and adaptive immunity.

7.3 Conclusion

In conclusion, the work presented in this thesis shows, for the first time that *T. carassii* induces expression of an acute phase response during both the acute and chronic phases of infection, and that they play a role in this host-parasite interaction. Two major APPs (CRP and SAA) were cloned, expressed and recombinant proteins functionally characterized. I believe that my research has contributed significantly to our understanding of an important aspect of innate immunity of bony fish, the acute phase response during a pathogenic infection. My research has also identified potential molecules that may be used for monitoring the health status of fish exposed to different biotic and abiotic stressors.

REFERENCES

[1] C. Uribe, H. Folch, R. Enriquez, G. Moran, Innate and adaptive immunity in teleost fish: a review., Veterinární Medicína. 56 (2011) 486–503.

[2] C.J. Bayne, L. Gerwick, The acute phase response and innate immunity of fish, Dev. Comp. Immunol. 25 (2001) 725–743. doi:10.1016/S0145-305X(01)00033-7.

[3] E. Gruys, M.J.M. Toussaint, T.A. Niewold, S.J. Koopmans, Acute phase reaction and acute phase proteins, J. Zhejiang Univ. Sci. B. 6 (2005) 1045–1056. doi:10.1631/jzus.2005.B1045.

[4] S. Jain, V. Gautam, S. Naseem, Acute-phase proteins: As diagnostic tool, J. Pharm. Bioallied Sci. 3 (2011) 118–127. doi:10.4103/0975-7406.76489.

[5] J.G. Skinner, for the E.C.A.G. on A.P. Proteins, International standardization of acute phase proteins, Vet. Clin. Pathol. 30 (2001) 2–7. doi:10.1111/j.1939-165X.2001.tb00248.x.

[6] E. Peatman, P. Baoprasertkul, J. Terhune, P. Xu, S. Nandi, H. Kucuktas, et al., Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium, Dev. Comp. Immunol. 31 (2007) 1183–1196. doi:10.1016/j.dci.2007.03.003.

[7] K. Buchmann, Impact and control of protozoan parasites in maricultured fishes, Parasitology. 142 (2015) 168–177. doi:10.1017/S003118201300005X.

[8] S.R.. Jones, The occurrence and mechanisms of innate immunity against parasites in fish, Dev. Comp. Immunol. 25 (2001) 841–852. doi:10.1016/S0145-305X(01)00039-8.

[9] A. Oladiran, M. Belosevic, *Trypanosoma carassii* calreticulin binds host complement component C1q and inhibits classical complement pathway-mediated lysis, Dev. Comp. Immunol. 34 (2010) 396–405. doi:10.1016/j.dci.2009.11.005.

[10] F. Aguero, V. Campo, L. Cremona, A. Jager, J.M. Di Noia, P. Overath, et al., Gene discovery in the freshwater fish parasite <I>Trypanosoma carassii</i>: Identification of trans-sialidase-like and mucin-like genes, Infect. Immun. 70 (2002) 7140–7144. doi:10.1128/IAI.70.12.7140-7144.2002.

[11] N.F. Neumann, D.R. Barreda, M. Belosevic, Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures, Fish Shellfish Immunol. 10 (2000) 1–20. doi:10.1006/fsim.1999.0221.

[12] D.A. Plouffe, Immunity of goldfish *Carassius auratus* (L.) to *Trypanosoma danilewskyi* (Laveran and Mesnil, 1904), Doctoral Dissertation, University of Alberta, 2005.

[13] T. Boehm, Evolution of vertebrate immunity, Curr. Biol. 22 (2012) R722–R732. doi:10.1016/j.cub.2012.07.003.

[14] C.A. Janeway, R. Medzhitov, Innate immune recognition, Annu. Rev. Immunol. 20 (2002) 197–216. doi:10.1146/annurev.immunol.20.083001.084359.

[15] M.A. Esteban, An Overview of the Immunological Defenses in Fish Skin, Int. Sch. Res. Not. 2012 (2012) 1–29. doi:10.5402/2012/853470.

[16] C.D. Anderson, R.J. Roberts, A comparison of the effects of temperature on wound healing in a tropical and a temperate teleost, J. Fish Biol. 7 (1975) 173–182. doi:10.1111/j.1095-8649.1975.tb04588.x.

[17] D.K. Fontenot, D.L. Neiffer, Wound management in teleost fish: biology of the healing process, evaluation, and treatment, Veterinary Clin. North Am. Exot. Anim. Pract. 7 (2004) 57–86.
[18] B.H. Beck, E. Peatman, Mucosal Health in Aquaculture, Academic Press, 2015.
[19] D.G. Elliott, Chapter 5 - Integumentary System, in: G.K. Ostrander (Ed.), Lab. Fish, Academic Press, London, 2000: pp. 95–108.
[20] P.T.K. Woo, K. Buchmann, Fish Parasites: Pathobiology and Protection, CABI, 2012.
[21] J.L. Hodgkinson, D. Bucke, B. Austin, Uptake of the fish pathogen, *Aeromonas salmonicida*, by rainbow trout (*Salmo gairdneri* L.), FEMS Microbiol. Lett. 40 (1987) 207–210. doi:10.1111/j.1574-6968.1987.tb02026.x.
[22] E. Tobback, K. Hermans, A. Decostere, W. Van den Broeck, F. Haesebrouck, K. Chiers, Interactions of virulent and avirulent *Yersinia ruckeri* strains with isolated gill arches and intestinal explants of rainbow trout *Oncorhynchus mykiss*, Dis. Aquat. Organ. 90 (2010) 175–179. doi:10.3354/dao02230.
[23] E. Tobback, A. Decostere, K. Hermans, F. Haesebrouck, K. Chiers, *Yersinia ruckeri* infections in salmonid fish, J. Fish Dis. 30 (2007) 257–268. doi:10.1111/j.1365-2761.2007.00816.x.
[24] H.W. Dickerson, R.C. Findly, Immunity to Ichthyophthirius infections in fish: a synopsis, Dev. Comp. Immunol. 43 (2014) 290–299. doi:10.1016/j.dci.2013.06.004.
[25] M.B. Adams, B.F. Nowak, Distribution and structure of lesions in the gills of Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease, J. Fish Dis. 24 (2001) 535–542. doi:10.1046/j.1365-2761.2001.00330.x.
[26] C.M. Press, Ø. Evensen, The morphology of the immune system in teleost fishes, Fish Shellfish Immunol. 9 (1999) 309–318. doi:10.1006/fsim.1998.0181.
[27] E.O. Koppang, A. Kvellestad, U. Fischer, 5 - Fish mucosal immunity: gill, in: B.H.B. Peatman (Ed.), Mucosal Health Aquac., Academic Press, San Diego, 2015: pp. 93–133.
[28] R.N. Morrison, E.O. Koppang, I. Hordvik, B.F. Nowak, MHC class II+ cells in the gills of Atlantic salmon (*Salmo salar* L.) affected by amoebic gill disease, Vet. Immunol. Immunopathol. 109 (2006) 297–303. doi:10.1016/j.vetimm.2005.08.026.
[29] I. Salinas, D. Parra, 6 - Fish mucosal immunity: intestine, in: B.H.B. Peatman (Ed.), Mucosal Health Aquac., Academic Press, San Diego, 2015: pp. 135–170.
[30] C.J. Secombes, A.E. Ellis, The Immunology of Teleosts, in: R.J.R.B., (Glasgow), FRCVS, FRCPath, FSB FRSE (Ed.), Fish Pathol., Wiley-Blackwell, 2012: pp. 144–166.
[31] T. Pérez, J.L. Balcázar, I. Ruiz-Zarzuela, N. Halaihel, D. Vendrell, I. de Blas, et al., Host–microbiota interactions within the fish intestinal ecosystem, Mucosal Immunol. 3 (2010) 355–360. doi:10.1038/mi.2010.12.
[32] B. Magnadóttir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2006) 137–151. doi:10.1016/j.fsi.2004.09.006.
[33] T.S. Peterson, 3 - Overview of mucosal structure and function in teleost fishes, in: B.H.B. Peatman (Ed.), Mucosal Health Aquac., Academic Press, San Diego, 2015: pp. 55–65.

[34] K. Buchmann, J. Bresciani, Microenvironment of *Gyrodactylus derjavini* on rainbow trout *Oncorhynchus mykiss*: association between mucous cell density in skin and site selection, Parasitol. Res. 84 (1998) 17–24.
[35] I. Estensoro, V. Jung-Schroers, P. Álvarez-Pellitero, D. Steinhagen, A. Sitjà-Bobadilla, Effects of *Enteromyxum leei* (Myxozoa) infection on gilthead sea bream (*Sparus aurata*) (Teleostei) intestinal mucus: glycoprotein profile and bacterial adhesion, Parasitol. Res. 112 (2012) 567–576. doi:10.1007/s00436-012-3168-3.
[36] R. Castro, C. Tafalla, 2 - Overview of fish immunity, in: B.H.B. Peatman (Ed.), Mucosal Health Aquac., Academic Press, San Diego, 2015: pp. 3–54.
[37] T. Nakanishi, H. Toda, Y. Shibasaki, T. Somamoto, Cytotoxic T cells in teleost fish, Dev. Comp. Immunol. 35 (2011) 1317–1323. doi:10.1016/j.dci.2011.03.033.
[38] J.A. Yoder, Investigating the morphology, function and genetics of cytotoxic cells in bony fish, Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 138 (2004) 271–280. doi:10.1016/j.cca.2004.03.008.
[39] D.L. Evans, L. Jaso-Friedmann, E.E. Smith, A. St John, H.S. Koren, D.T. Harris, Identification of a putative antigen receptor on fish nonspecific cytotoxic cells with monoclonal antibodies, J. Immunol. Baltim. Md 1950. 141 (1988) 324–332.
[40] D.L. Evans, L. Jaso-Friedmann, Nonspecific cytotoxic cells as effectors of immunity in fish, Annu. Rev. Fish Dis. 2 (1992) 109–121. doi:10.1016/0959-8030(92)90059-7.
[41] L. Shen, T.B. Stuge, H. Zhou, M. Khayat, K.S. Barker, S.M.A. Quiniou, et al., Channel catfish cytotoxic cells: a mini-review, Dev. Comp. Immunol. 26 (2002) 141–149. doi:10.1016/S0145-305X(01)00056-8.
[42] S.S. Graves, D.L. Evans, D.L. Dawe, Antiprotozoan activity of nonspecific cytotoxic cells (NCC) from the channel catfish (*Ictalurus punctatus*)., J. Immunol. 134 (1985) 78–85.
[43] L. Shen, T.B. Stuge, E. Bengtén, M. Wilson, V.G. Chinchar, J.P. Naftel, et al., Identification and characterization of clonal NK-like cells from channel catfish (*Ictalurus punctatus*), Dev. Comp. Immunol. 28 (2004) 139–152.
[44] T. Nakanishi, U. Fischer, J.M. Dijkstra, S. Hasegawa, T. Somamoto, N. Okamoto, et al., Cytotoxic T cell function in fish, Dev. Comp. Immunol. 26 (2002) 131–139.
[45] T. Nakanishi, K. Aoyagi, C. Xia, J.M. Dijkstra, M. Ototake, Specific cell-mediated immunity in fish, Vet. Immunol. Immunopathol. 72 (1999) 101–109. doi:10.1016/S0165-2427(99)00122-1.
[46] R. Castro, D. Bernard, M.P. Lefranc, A. Six, A. Benmansour, P. Boudinot, T cell diversity and TcR repertoires in teleost fish, Fish Shellfish Immunol. 31 (2011) 644–654. doi:10.1016/j.fsi.2010.08.016.
[47] T. Somamoto, T. Nakanishi, M. Nakao, Identification of anti-viral cytotoxic effector cells in the ginbuna crucian carp, *Carassius auratus langsdorfii*, Dev. Comp. Immunol. 39 (2013) 370–377. doi:10.1016/j.dci.2012.11.001.
[48] H. Toda, K. Araki, T. Moritomo, T. Nakanishi, Perforin-dependent cytotoxic mechanism in killing by CD8 positive T cells in ginbuna crucian carp, *Carassius auratus langsdorfii*, Dev. Comp. Immunol. 35 (2011) 88–93. doi:10.1016/j.dci.2010.08.010.

[49] M. Yamasaki, K. Araki, T. Nakanishi, C. Nakayasu, A. Yamamoto, Role of CD4(+) and CD8α(+) T cells in protective immunity against *Edwardsiella tarda* infection of ginbuna crucian carp, *Carassius auratus langsdorfii*, Fish Shellfish Immunol. 36 (2014) 299–304. doi:10.1016/j.fsi.2013.11.016.

[50] A.J. Ainsworth, Fish granulocytes: Morphology, distribution, and function, Annu. Rev. Fish Dis. 2 (1992) 123–148. doi:10.1016/0959-8030(92)90060-B.

[51] O.B. Reite, The mast cell nature of granule cells in the digestive tract of the pike, *Esox lucius*: similarity to mammalian mucosal mast cells and globule leucocytes, Fish Shellfish Immunol. 6 (1996) 363–369. doi:10.1006/fsim.1996.0036.

[52] O.B. Reite, Ø. Evensen, Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells, Fish Shellfish Immunol. 20 (2006) 192–208. doi:10.1016/j.fsi.2005.01.012.

[53] C. Garavini, P. Martelli, Alkaline phosphatase and peroxidase in goldfish (*Carassius auratus*) leukocytes, Basic Appl. Histochem. 25 (1981) 133–139.

[54] N. Fijan, Morphogenesis of blood cell lineages in channel catfish, J. Fish Biol. 60 (2002) 999–1014. doi:10.1111/j.1095-8649.2002.tb02424.x.

[55] K.M. Balla, G. Lugo-Villarino, J.M. Spitsbergen, D.L. Stachura, Y. Hu, K. Bañuelos, et al., Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants, Blood. 116 (2010) 3944–3954. doi:10.1182/blood-2010-03-267419.

[56] J.T. Dobson, J. Seibert, E.M. Teh, S. Da'as, R.B. Fraser, B.H. Paw, et al., Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination, Blood. 112 (2008) 2969–2972. doi:10.1182/blood-2008-03-145011.

[57] A. Sfacteria, M. Brines, U. Blank, The mast cell plays a central role in the immune system of teleost fish, Mol. Immunol. 63 (2015) 3–8. doi:10.1016/j.molimm.2014.02.007.

[58] M. Tavares-Dias, Cytochemical method for staining fish basophils, J. Fish Biol. 69 (2006) 312–317. doi:10.1111/j.1095-8649.2006.01106.x.

[59] E. Bielek, J. Bigaj, M. Chadzińska, B. Płytycz, Depletion of head kidney neutrophils and cells with basophilic granules during peritoneal inflammation in the goldfish, *Carassius auratus*, Folia Biol. (Praha). 47 (1999) 33–42.

[60] B.A. Katzenback, M. Belosevic, Isolation and functional characterization of neutrophil-like cells, from goldfish (*Carassius auratus* L.) kidney, Dev. Comp. Immunol. 33 (2009) 601–611. doi:10.1016/j.dci.2008.10.011.

[61] H. Kodama, K. Tijiwa, T. Moritomo, T. Nakanishi, Granulocyte responses to experimental injection of live and formalin-killed bacteria in carp (*Cyprinus carpio*), Vet. Immunol. Immunopathol. 90 (2002) 101–105.

[62] N.F. Neumann, J.L. Stafford, D. Barreda, A.J. Ainsworth, M. Belosevic, Antimicrobial mechanisms of fish phagocytes and their role in host defense, Dev. Comp. Immunol. 25 (2001) 807–825. doi:10.1016/S0145-305X(01)00037-4.

[63] K.M. Henry, C.A. Loynes, M.K.B. Whyte, S.A. Renshaw, Zebrafish as a model for the study of neutrophil biology, J. Leukoc. Biol. 94 (2013) 633–642. doi:10.1189/jlb.1112594.

[64] D.R. Barreda, N.F. Neumann, M. Belosevic, Flow cytometric analysis of PKH26-labeled goldfish kidney-derived macrophages, Dev. Comp. Immunol. 24 (2000) 395–406.
[65] L. Grayfer, M. Belosevic, Molecular characterization, expression and functional analysis of goldfish (*Carassius aurutus* L.) interferon gamma, Dev. Comp. Immunol. 33 (2009) 235–246. doi:10.1016/j.dci.2008.09.001.
[66] J. Zou, C.J. Secombes, Teleost fish interferons and their role in immunity, Dev. Comp. Immunol. 35 (2011) 1376–1387. doi:10.1016/j.dci.2011.07.001.
[67] M. Joerink, M. Forlenza, C.M.S. Ribeiro, B.J. de Vries, H.F.J. Savelkoul, G.F. Wiegertjes, Differential macrophage polarisation during parasitic infections in common carp (*Cyprinus carpio* L.), Fish Shellfish Immunol. 21 (2006) 561–571. doi:10.1016/j.fsi.2006.03.006.
[68] R. Savan, M. Sakai, Genomics of fish cytokines, Comp. Biochem. Physiol. Part D Genomics Proteomics. 1 (2006) 89–101. doi:10.1016/j.cbd.2005.08.005.
[69] L. Grayfer, J.G. Walsh, M. Belosevic, Characterization and functional analysis of goldfish (*Carassius auratus* L.) tumor necrosis factor-alpha, Dev. Comp. Immunol. 32 (2008) 532–543. doi:10.1016/j.dci.2007.09.009.
[70] L. Grayfer, The analysis of cytokine regulation of macrophage antimicrobial responses of the goldfish (*Carassius auratus* L.), Doctoral Disseration, University of Alberta, 2011.
[71] L. Grayfer, M. Belosevic, Cytokine regulation of teleost inflammatory responses, in: H. Turker (Ed.), New Adv. Contrib. Fish Biol., InTech, 2012. http://www.intechopen.com/books/new-advances-and-contributions-to-fish-biology/cytokine-regulation-of-teleost-inflammatory-responses (accessed August 14, 2015).
[72] M. Watts, B. Munday, C. Burke, Immune responses of teleost fish, Aust. Vet. J. 79 (2001) 570–574. doi:10.1111/j.1751-0813.2001.tb10753.x.
[73] S. Saurabh, P.K. Sahoo, Lysozyme: an important defence molecule of fish innate immune system, Aquac. Res. 39 (2008) 223–239. doi:10.1111/j.1365-2109.2007.01883.x.
[74] L. Tort, Balasch, C., Mackenzie, S., Fish immune system. A crossroads between innate and adaptive responses, Immunologia. 22 (2003) 277–286.
[75] S.K. Whyte, The innate immune response of finfish – A review of current knowledge, Fish Shellfish Immunol. 23 (2007) 1127–1151. doi:10.1016/j.fsi.2007.06.005.
[76] C. Cray, J. Zaias, N.H. Altman, Acute phase response in animals: A review, Comp. Med. 59 (2009) 517–526.
[77] C.A. Dinarello, Interleukin-1 and the pathogenesis of the acute-phase response, N. Engl. J. Med. 311 (1984) 1413–1418. doi:10.1056/NEJM198411293112205.
[78] H. Moshage, Cytokines and the hepatic acute phase response, J. Pathol. 181 (1997) 257–266. doi:10.1002/(SICI)1096-9896(199703)181:3<257::AID-PATH756>3.0.CO;2-U.
[79] D.M. Steel, A.S. Whitehead, The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein, Immunol. Today. 15 (1994) 81–88. doi:10.1016/0167-5699(94)90138-4.

[80] H. Baumann, J. Gauldie, The acute phase response, Immunol. Today. 15 (1994) 74–80. doi:10.1016/0167-5699(94)90137-6.
[81] P. Alvarez-Pellitero, Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects, Vet. Immunol. Immunopathol. 126 (2008) 171–198. doi:10.1016/j.vetimm.2008.07.013.
[82] Rv. Kumar, Rm. Kumar, N. Pradeep Kumar, N. Ananthakrishnan, Diagnostic value of C-reactive protein in suspected acute appendicitis - A prospective case control study, Indian J. Med. Sci. 65 (2011) 399. doi:10.4103/0019-5359.108956.
[83] D.H. McGuinness, P.K. Dehal, R.J. Pleass, Pattern recognition molecules and innate immunity to parasites, Trends Parasitol. 19 (2003) 312–319.
[84] I. Kushner, D.L. Rzewnicki, The acute phase response: general aspects, Baillières Clin. Rheumatol. 8 (1994) 513–530.
[85] J.B. Covert, W.W. Reynolds, Survival value of fever in fish, Nature. 267 (1977) 43–45. doi:10.1038/267043a0.
[86] A. Gräns, M. Rosengren, L. Niklasson, M. Axelsson, Behavioural fever boosts the inflammatory response in rainbow trout Oncorhynchus mykiss, J. Fish Biol. 81 (2012) 1111–1117. doi:10.1111/j.1095-8649.2012.03333.x.
[87] E.D. Stevens, A.M. Sutterlin, Heat transfer between fish and ambient water, J. Exp. Biol. 65 (1976) 131–145.
[88] F. Ceciliani, A. Giordano, V. Spagnolo, The systemic reaction during inflammation: The acute-phase proteins, Protein Pept. Lett. 9 (2002) 211–223. doi:10.2174/0929866023408779.
[89] H. Murata, N. Shimada, M. Yoshioka, Current research on acute phase proteins in veterinary diagnosis: an overview, Vet. J. 168 (2004) 28–40. doi:10.1016/S1090-0233(03)00119-9.
[90] A. Koj, Termination of Acute-Phase Response: Role of Some Cytokines and Anti-Inflammatory Drugs, Gen. Pharmacol. Vasc. Syst. 31 (1998) 9–18. doi:10.1016/S0306-3623(97)00435-7.
[91] M.D. Glibetic, H. Baumann, Influence of chronic inflammation on the level of mRNA for acute-phase reactants in the mouse liver, J. Immunol. Baltim. Md 1950. 137 (1986) 1616–1622.
[92] P.H. Sampaio, O.L.F. Junior, L.C. Marques, R.Z. Machado, P. de A. Barnabé, M.R. André, et al., Acute-phase protein behavior in dairy cattle herd naturally infected with *Trypanosoma vivax*, Vet. Parasitol. 211 (2015) 141–145. doi:10.1016/j.vetpar.2015.05.014.
[93] C. Gabay, I. Kushner, Acute-phase proteins and other systemic responses to inflammation, N. Engl. J. Med. 340 (1999) 448–454. doi:10.1056/NEJM199902113400607.
[94] A. Mantovani, C. Garlanda, A. Doni, B. Bottazzi, Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3, J. Clin. Immunol. 28 (2007) 1–13. doi:10.1007/s10875-007-9126-7.
[95] M.B. Pepys, G.M. Hirschfield, C-reactive protein: a critical update, J. Clin. Invest. 111 (2003) 1805–1812. doi:10.1172/JCI18921.

[96] J. Paul Simons, J.M. Loeffler, R. Al-Shawi, S. Ellmerich, W.L. Hutchinson, G.A. Tennent, et al., C-reactive protein is essential for innate resistance to pneumococcal infection, Immunology. 142 (2014) 414–420. doi:10.1111/imm.12266.
[97] T.W.D. Clos, C. Mold, C-reactive protein, Immunol. Res. 30 (2004) 261–277. doi:10.1385/IR:30:3:261.
[98] C. Garlanda, B. Bottazzi, A. Bastone, A. Mantovani, Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility, Annu. Rev. Immunol. 23 (2005) 337–366. doi:10.1146/annurev.immunol.23.021704.115756.
[99] J.R. Cartwright, H.A. Tharia, I. Burns, A.K. Shrive, D. Hoole, T.J. Greenhough, Isolation and characterisation of pentraxin-like serum proteins from the common carp *Cyprinus carpio*, Dev. Comp. Immunol. 28 (2004) 113–125. doi:10.1016/S0145-305X(03)00123-X.
[100] A.E. Ellis, Innate host defense mechanisms of fish against viruses and bacteria, Dev. Comp. Immunol. 25 (2001) 827–839.
[101] C.S. Carlson, S.F. Aldred, P.K. Lee, R.P. Tracy, S.M. Schwartz, M. Rieder, et al., Polymorphisms within the C-Reactive Protein (CRP) promoter region are associated with plasma CRP levels, Am. J. Hum. Genet. 77 (2005) 64–77. doi:10.1086/431366.
[102] A.J. Szalai, M.A. McCrory, G.S. Cooper, J. Wu, R.P. Kimberly, Association between baseline levels of C-reactive protein (CRP) and a dinucleotide repeat polymorphism in the intron of the CRP gene, Genes Immun. 3 (2002) 14–19. doi:10.1038/sj.gene.6363820.
[103] J.M. Kilpatrick, J.E. Volanakis, Molecular genetics, structure, and function of C-reactive protein, Immunol. Res. 10 (1991) 43–53. doi:10.1007/BF02918166.
[104] A.J. Szalai, A. Agrawal, T.J. Greenhough, J.E. Volanakis, C-reactive protein: structural biology and host defense function, Clin. Chem. Lab. Med. CCLM FESCC. 37 (1999) 265–270. doi:10.1515/CCLM.1999.046.
[105] J.E. Volanakis, Human C-reactive protein: expression, structure, and function, Mol. Immunol. 38 (2001) 189–197. doi:10.1016/S0161-5890(01)00042-6.
[106] J.E. Volanakis, M.H. Kaplan, Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide, Proc. Soc. Exp. Biol. Med. 136 (1971) 612–614.
[107] T.W. Du Clos, Function of C-reactive protein, Ann. Med. 32 (2000) 274–278.
[108] S.P. Ballou, G. Lozanski, Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein, Cytokine. 4 (1992) 361–368.
[109] B. Galve-de Rochemonteix, K. Wiktorowicz, I. Kushner, J.M. Dayer, C-reactive protein increases production of IL-1 alpha, IL-1 beta, and TNF-alpha, and expression of mRNA by human alveolar macrophages, J. Leukoc. Biol. 53 (1993) 439–445.
[110] S. Black, I. Kushner, D. Samols, C-reactive Protein, J. Biol. Chem. 279 (2004) 48487–48490. doi:10.1074/jbc.R400025200.
[111] S.U. Eisenhardt, J.R. Thiele, H. Bannasch, G.B. Stark, K. Peter, C-reactive protein: how conformational changes influence inflammatory properties, Cell Cycle Georget. Tex. 8 (2009) 3885–3892.

[112] J.R. Thiele, J. Habersberger, D. Braig, Y. Schmidt, K. Goerendt, V. Maurer, et al., The dissociation of pentameric to monomeric C-reactive protein localizes and aggravates inflammation: *In vivo* proof of a powerful pro-Inflammatory mechanism and a new anti-inflammatory strategy, Circulation. 1 (2014) 35–50. doi:10.1161/CIRCULATIONAHA.113.007124.

[113] A.J. Szalai, S. Nataf, X.-Z. Hu, S.R. Barnum, Experimental allergic encephalomyelitis is inhibited in transgenic mice expressing human C-reactive protein, J. Immunol. Baltim. Md 1950. 168 (2002) 5792–5797.

[114] M. Li, C. Chen, J. Li, L. Sun, The C-reactive protein of tongue sole *Cynoglossus semilaevis* is an acute phase protein that interacts with bacterial pathogens and stimulates the antibacterial activity of peripheral blood leukocytes, Fish Shellfish Immunol. 34 (2013) 623–631. doi:10.1016/j.fsi.2012.12.001.

[115] P.T. Le, M.T. Muller, R.F. Mortensen, Acute phase reactants of mice. I. Isolation of serum amyloid P-component (SAP) and its induction by a monokine, J. Immunol. Baltim. Md 1950. 129 (1982) 665–672.

[116] S. Sinha, C. Mandal, Microheterogeneity of C-reactive protein in the sera of fish *Labeo rohita* induced by metal pollutants, Biochem. Biophys. Res. Commun. 226 (1996) 681–687. doi:10.1006/bbrc.1996.1414.

[117] L.A.H. Mohamed-Jawad, A. Rabu, R. Mohamed, A. Mohd-Adnan, Phylogenetic characterization and the expression of recombinant C-reactive protein from the Asian seabass (*Lates calcarifer*), Aquaculture. 338–341 (2012) 13–22. doi:10.1016/j.aquaculture.2011.12.047.

[118] V. Lund, J.A. Olafsen, Changes in serum concentration of a serum amyloid P-like pentraxin in Atlantic salmon, *Salmo salar* L., during infection and inflammation, Dev. Comp. Immunol. 23 (1999) 61–70. doi:10.1016/S0145-305X(98)00038-X.

[119] M. Murata, M. Onuma, H. Kodama, Isolation and characterization of rainbow trout (*Oncorhynchus mykiss*) serum amyloid P component (SAP), J. Vet. Med. Sci. Jpn. Soc. Vet. Sci. 56 (1994) 661–665.

[120] D.T. Huong Giang, E. Van Driessche, I. Vandenberghe, B. Devreese, S. Beeckmans, Isolation and characterization of SAP and CRP, two pentraxins from *Pangasianodon (Pangasius) hypophthalmus*, Fish Shellfish Immunol. 28 (2010) 743–753. doi:10.1016/j.fsi.2010.01.007.

[121] B. Gisladottir, S. Gudmundsdottir, L. Brown, Z.O. Jonsson, B. Magnadottir, Isolation of two C-reactive protein homologues from cod (*Gadus morhua* L.) serum, Fish Shellfish Immunol. 26 (2009) 210–219. doi:10.1016/j.fsi.2008.03.015.

[122] M.T. Cook, P.J. Hayball, B.F. Nowak, J.D. Hayball, The opsonising activity of a pentraxin-like protein isolated from snapper (*Pagrus auratus*, Sparidae) serum, Dev. Comp. Immunol. 29 (2005) 703–712. doi:10.1016/j.dci.2004.05.009.

[123] N. Pionnier, M. Adamek, J.J. Miest, S.J. Harris, M. Matras, K.Ł. Rakus, et al., C-reactive protein and complement as acute phase reactants in common carp *Cyprinus carpio* during CyHV-3 infection, Dis. Aquat. Organ. 109 (2014) 187–199. doi:10.3354/dao02727.

[124] K.K. Eklund, K. Niemi, P.T. Kovanen, Immune functions of serum amyloid A, Crit. Rev. Immunol. 32 (2012) 335–348.

[125] C.M. Uhlar, A.S. Whitehead, Serum amyloid A, the major vertebrate acute-phase reactant, Eur. J. Biochem. FEBS. 265 (1999) 501–523.
[126] S. Urieli-Shoval, R.P. Linke, Y. Matzner, Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states, Curr. Opin. Hematol. 7 (2000) 64–69.
[127] N. Cheng, R. He, J. Tian, P.P. Ye, R.D. Ye, Cutting Edge: TLR2 is a functional receptor for acute-phase serum amyloid A, J. Immunol. Baltim. Md 1950. 181 (2008) 22–26.
[128] H.Y. Lee, M.-K. Kim, K.S. Park, E.H. Shin, S.H. Jo, S.D. Kim, et al., Serum amyloid A induces contrary immune responses via formyl peptide receptor-like 1 in human monocytes, Mol. Pharmacol. 70 (2006) 241–248. doi:10.1124/mol.105.022103.
[129] C. Song, K. Hsu, E. Yamen, W. Yan, J. Fock, P.K. Witting, et al., Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes, Atherosclerosis. 207 (2009) 374–383. doi:10.1016/j.atherosclerosis.2009.05.007.
[130] R. Kisilevsky, P.N. Manley, Acute-phase serum amyloid A: Perspectives on its physiological and pathological roles, Amyloid. 19 (2012) 5–14. doi:10.3109/13506129.2011.654294.
[131] E.P. Benditt, N. Eriksen, Amyloid protein SAA is associated with high density lipoprotein from human serum., Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 4025–4028.
[132] E.P. Benditt, N. Eriksen, Chemical classes of amyloid substance, Am. J. Pathol. 65 (1971) 231–252.
[133] C.M. Uhlar, C.J. Burgess, P.M. Sharp, A.S. Whitehead, Evolution of the serum amyloid A (SAA) protein superfamily, Genomics. 19 (1994) 228–235. doi:10.1006/geno.1994.1052.
[134] A.S. Whitehead, M.C. de Beer, D.M. Steel, M. Rits, J.M. Lelias, W.S. Lane, et al., Identification of novel members of the serum amyloid A protein superfamily as constitutive apolipoproteins of high density lipoprotein, J. Biol. Chem. 267 (1992) 3862–3867.
[135] J.T. Guo, C.E. Aldrich, W.S. Mason, J.C. Pugh, Characterization of serum amyloid A protein mRNA expression and secondary amyloidosis in the domestic duck, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 14548–14553.
[136] A. Rebl, T. Goldammer, U. Fischer, B. Köllner, H.-M. Seyfert, Characterization of two key molecules of teleost innate immunity from rainbow trout (*Oncorhynchus mykiss*): MyD88 and SAA, Vet. Immunol. Immunopathol. 131 (2009) 122–126. doi:10.1016/j.vetimm.2009.03.006.
[137] P. Santiago, J.L. Roig-López, C. Santiago, J.E. García-Arrarás, Serum amyloid A protein in an echinoderm: its primary structure and expression during intestinal regeneration in the sea cucumber *Holothuria glaberrima*, J. Exp. Zool. 288 (2000) 335–344. doi:10.1002/1097-010X(20001215)288:4<335::AID-JEZ6>3.0.CO;2-1.
[138] Patel, Fellowes, Coade, Woo, Human Serum amyloid A has cytokine-like properties, Scand. J. Immunol. 48 (1998) 410–418. doi:10.1046/j.1365-3083.1998.00394.x.

[139] H.Y. Lee, S.D. Kim, J.W. Shim, S.Y. Lee, H. Lee, K.-H. Cho, et al., Serum Amyloid A induces CCL2 production via formyl peptide receptor-like 1-mediated signaling in human monocytes, J. Immunol. 181 (2008) 4332–4339. doi:10.4049/jimmunol.181.6.4332.
[140] H.Y. Lee, M.-K. Kim, K.S. Park, Y.H. Bae, J. Yun, J.-I. Park, et al., Serum amyloid A stimulates matrix-metalloproteinase-9 upregulation via formyl peptide receptor like-1-mediated signaling in human monocytic cells, Biochem. Biophys. Res. Commun. 330 (2005) 989–998. doi:10.1016/j.bbrc.2005.03.069.
[141] R. Badolato, J.M. Wang, W.J. Murphy, A.R. Lloyd, D.F. Michiel, L.L. Bausserman, et al., Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes, J. Exp. Med. 180 (1994) 203–209.
[142] R.L. He, J. Zhou, C.Z. Hanson, J. Chen, N. Cheng, R.D. Ye, Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor 2, Blood. 113 (2009) 429–437. doi:10.1182/blood-2008-03-139923.
[143] L. Xu, R. Badolato, W.J. Murphy, D.L. Longo, M. Anver, S. Hale, et al., A novel biologic function of serum amyloid A. Induction of T lymphocyte migration and adhesion., J. Immunol. 155 (1995) 1184–1190.
[144] S. Sandri, D. Rodriguez, E. Gomes, H.P. Monteiro, M. Russo, A. Campa, Is serum amyloid A an endogenous TLR4 agonist?, J. Leukoc. Biol. 83 (2008) 1174–1180. doi:10.1189/jlb.0407203.
[145] L. Björkman, J. Karlsson, A. Karlsson, M.-J. Rabiet, F. Boulay, H. Fu, et al., Serum amyloid A mediates human neutrophil production of reactive oxygen species through a receptor independent of formyl peptide receptor like-1, J. Leukoc. Biol. 83 (2008) 245–253. doi:10.1189/jlb.0607-408.
[146] I.N. Baranova, A.V. Bocharov, T.G. Vishnyakova, R. Kurlander, Z. Chen, D. Fu, et al., CD36 is a novel serum amyloid A (SAA) receptor mediating SAA binding and SAA-induced signaling in human and rodent cells, J. Biol. Chem. 285 (2010) 8492–8506. doi:10.1074/jbc.M109.007526.
[147] M. Chen, H. Zhou, N. Cheng, F. Qian, R.D. Ye, Serum amyloid A1 isoforms display different efficacy at Toll-like receptor 2 and formyl peptide receptor 2, Immunobiology. 219 (2014) 916–923. doi:10.1016/j.imbio.2014.08.002.
[148] K. Niemi, L. Teirilä, J. Lappalainen, K. Rajamäki, M.H. Baumann, K. Öörni, et al., Serum Amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway, J. Immunol. 186 (2011) 6119–6128. doi:10.4049/jimmunol.1002843.
[149] L.E. Jensen, M.P. Hiney, D.C. Shields, C.M. Uhlar, A.J. Lindsay, A.S. Whitehead, Acute phase proteins in salmonids: evolutionary analyses and acute phase response, J. Immunol. Baltim. Md 1950. 158 (1997) 384–392.
[150] K. Fujiki, D.-H. Shin, M. Nakao, T. Yano, Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1β, high affinity immunoglobulin E Fc receptor γ subunit and serum amyloid A, Fish Shellfish Immunol. 10 (2000) 229–242. doi:10.1006/fsim.1999.0253.

[151] B. Lin, S. Chen, Z. Cao, Y. Lin, D. Mo, H. Zhang, et al., Acute phase response in zebrafish upon *Aeromonas salmonicida* and *Staphylococcus aureus* infection: striking similarities and obvious differences with mammals, Mol. Immunol. 44 (2007) 295–301. doi:10.1016/j.molimm.2006.03.001.

[152] N. Mohd-Shaharuddin, A. Mohd-Adnan, B.-C. Kua, S. Nathan, Expression profile of immune-related genes in *Lates calcarifer* infected by *Cryptocaryon irritans*, Fish Shellfish Immunol. 34 (2013) 762–769. doi:10.1016/j.fsi.2012.11.052.

[153] J.P.J. Saeij, B.J. de Vries, G.F. Wiegertjes, The immune response of carp to *Trypanoplasma borreli*: kinetics of immune gene expression and polyclonal lymphocyte activation, Dev. Comp. Immunol. 27 (2003) 859–874. doi:10.1016/S0145-305X(03)00083-1.

[154] T.M. Tadiso, A. Krasnov, S. Skugor, S. Afanasyev, I. Hordvik, F. Nilsen, Gene expression analyses of immune responses in *Atlantic salmon* during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition, BMC Genomics. 12 (2011) 141. doi:10.1186/1471-2164-12-141.

[155] K. Vanya Ewart, J.C. Belanger, J. Williams, T. Karakach, S. Penny, S.C.M. Tsoi, et al., Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology, Dev. Comp. Immunol. 29 (2005) 333–347. doi:10.1016/j.dci.2004.08.004.

[156] F. Villarroel, A. Casado, J. Vásquez, E. Matamala, B. Araneda, R. Amthauer, et al., Serum amyloid A: A typical acute-phase reactant in rainbow trout?, Dev. Comp. Immunol. 32 (2008) 1160–1169. doi:10.1016/j.dci.2008.03.004.

[157] J.L. Beard, Iron biology in immune function, muscle metabolism and neuronal functioning, J. Nutr. 131 (2001) 568S–580S.

[158] M. Wick, W. Pinggera, P. Lehmann, M. Wick, W. Pinggera, P. Lehmann, Iron Metabolism, in: Clin. Asp. Lab. — Iron Metab. Anemias, Springer Vienna, 2011: pp. 3–16. http://www.springerlink.com/content/v315m1238247q484/abstract/

[159] G. Papanikolaou, K. Pantopoulos, Iron metabolism and toxicity, Toxicol. Appl. Pharmacol. 202 (2005) 199–211. doi:10.1016/j.taap.2004.06.021.

[160] C.P. Anderson, M. Shen, R.S. Eisenstein, E.A. Leibold, Mammalian iron metabolism and its control by iron regulatory proteins, Biochim. Biophys. Acta BBA - Mol. Cell Res. 1823 (2012) 1468–1483. doi:10.1016/j.bbamcr.2012.05.010.

[161] M. Nairz, D. Haschka, E. Demetz, G. Weiss, Iron at the interface of immunity and infection, Drug Metab. Transp. 5 (2014) 152. doi:10.3389/fphar.2014.00152.

[162] E.P. Skaar, The battle for iron between bacterial pathogens and their vertebrate hosts, PLoS Pathog. 6 (2010) e1000949. doi:10.1371/journal.ppat.1000949.

[163] G. Schreiber, A. Tsykin, A.R. Aldred, T. Thomas, W.P. Fung, P.W. Dickson, et al., The acute phase response in the rodent, Ann. N. Y. Acad. Sci. 557 (1989) 61–85.

[164] J.V. Neves, J.M. Wilson, P.N.S. Rodrigues, Transferrin and ferritin response to bacterial infection: the role of the liver and brain in fish, Dev. Comp. Immunol. 33 (2009) 848–857. doi:10.1016/j.dci.2009.02.001.

[165] J.L. Stafford, E.C. Wilson, M. Belosevic, Recombinant transferrin induces nitric oxide response in goldfish and murine macrophages, Fish Shellfish Immunol. 17 (2004) 171–185. doi:10.1016/j.fsi.2004.03.001.

[166] J.L. Stafford, N.F. Neumann, M. Belosevic, Products of proteolytic cleavage of transferrin induce nitric oxide response of goldfish macrophages, Dev. Comp. Immunol. 25 (2001) 101–115. doi:10.1016/S0145-305X(00)00048-3.
[167] J.L. Stafford, M. Belosevic, Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation, Dev. Comp. Immunol. 27 (2003) 539–554. doi:10.1016/S0145-305X(02)00138-6.
[168] L.A. Meyer, A.P. Durley, J.R. Prohaska, Z.L. Harris, Copper transport and metabolism are normal in aceruloplasminemic mice, J. Biol. Chem. 276 (2001) 36857–36861. doi:10.1074/jbc.M105361200.
[169] H. Liu, E. Peatman, W. Wang, J. Abernathy, S. Liu, H. Kucuktas, et al., Molecular responses of ceruloplasmin to *Edwardsiella ictaluri* infection and iron overload in channel catfish (*Ictalurus punctatus*), Fish Shellfish Immunol. 30 (2011) 992–997. doi:10.1016/j.fsi.2010.12.033.
[170] H. Miyajima, Aceruloplasminemia, an iron metabolic disorder, Neuropathol. Off. J. Jpn. Soc. Neuropathol. 23 (2003) 345–350.
[171] S. Cherukuri, N.A. Tripoulas, S. Nurko, P.L. Fox, Anemia and impaired stress-induced erythropoiesis in aceruloplasminemic mice, Blood Cells. Mol. Dis. 33 (2004) 346–355. doi:10.1016/j.bcmd.2004.07.003.
[172] I.M. Goldstein, H.B. Kaplan, H.S. Edelson, G. Weissmann, Ceruloplasmin: an acute phase reactant that scavenges oxygen-derived free radicals, Ann. N. Y. Acad. Sci. 389 (1982) 368–379.
[173] B. Harshavardhana, S.K. Rath, M. Mukherjee, Evaluation of serum ceruloplasmin in aggressive and chronic periodontitis patients, J. Indian Soc. Periodontol. 17 (2013) 333–337. doi:10.4103/0972-124X.115659.
[174] J.D. Gitlin, Transcriptional regulation of ceruloplasmin gene expression during inflammation, J. Biol. Chem. 263 (1988) 6281–6287.
[175] R. Scudiero, F. Trinchella, M. Riggio, E. Parisi, Structure and expression of genes involved in transport and storage of iron in red-blooded and hemoglobin-less antarctic notothenioids, Gene. 397 (2007) 1–11. doi:10.1016/j.gene.2007.03.003.
[176] B. Bakhautdin, M. Febbraio, E. Goksoy, C.A. de la Motte, M.F. Gulen, E.P. Childers, et al., Protective role of macrophage-derived ceruloplasmin in inflammatory bowel disease, Gut. 62 (2013) 209–219. doi:10.1136/gutjnl-2011-300694.
[177] J. Banha, L. Marques, R. Oliveira, M. de F. Martins, E. Paixão, D. Pereira, et al., Ceruloplasmin expression by human peripheral blood lymphocytes: a new link between immunity and iron metabolism, Free Radic. Biol. Med. 44 (2008) 483–492. doi:10.1016/j.freeradbiomed.2007.10.032.
[178] K.Y. Djoko, C.Y. Ong, M.J. Walker, A.G. McEwan, The Role of Copper and Zinc Toxicity in Innate Immune Defense against Bacterial Pathogens, J. Biol. Chem. 290 (2015) 18954–18961. doi:10.1074/jbc.R115.647099.
[179] A.P. Levy, R. Asleh, S. Blum, N.S. Levy, R. Miller-Lotan, S. Kalet-Litman, et al., Haptoglobin: basic and clinical aspects, Antioxid. Redox Signal. 12 (2009) 293–304. doi:10.1089/ars.2009.2793.
[180] J.W. Eaton, P. Brandt, J.R. Mahoney, J.T. Lee, Haptoglobin: a natural bacteriostat, Science. 215 (1982) 691–693.

[181] Marchand A, Galen RS, Van Lente F, The predictive value of serum haptoglobin in hemolytic disease, JAMA. 243 (1980) 1909–1911. doi:10.1001/jama.1980.03300450023014.
[182] N.K. Shinton, R.W. Richardson, J.D.F. Williams, Diagnostic value of serum haptoglobin, J. Clin. Pathol. 18 (1965) 114–118.
[183] K.D. McClatchey, Clinical Laboratory Medicine, Lippincott Williams & Wilkins, 2002.
[184] S. Rogerson, What Is the relationship between haptoglobin, malaria, and anaemia?, PLoS Med. 3 (2006) e200. doi:10.1371/journal.pmed.0030200.
[185] I.K.E. Quaye, F.A. Ekuban, B.Q. Goka, V. Adabayeri, J.A.L. Kurtzhals, B. Gyan, et al., Haptoglobin 1-1 is associated with susceptibility to severe *Plasmodium falciparum* malaria, Trans. R. Soc. Trop. Med. Hyg. 94 (2000) 216–219. doi:10.1016/S0035-9203(00)90281-5.
[186] K.M. Huntoon, Y. Wang, C.A. Eppolito, K.W. Barbour, F.G. Berger, P.A. Shrikant, et al., The acute phase protein haptoglobin regulates host immunity, J. Leukoc. Biol. 84 (2008) 170–181. doi:10.1189/jlb.0208100.
[187] L. Gerwick, G. Corley-Smith, C.J. Bayne, Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus, Fish Shellfish Immunol. 22 (2007) 157–171. doi:10.1016/j.fsi.2006.04.003.
[188] J.D.H.E. Jayasinghe, D.A.S. Elvitigala, I. Whang, B.-H. Nam, J. Lee, Molecular characterization of two immunity-related acute-phase proteins: Haptoglobin and serum amyloid A from black rockfish (*Sebastes schlegeli*), Fish Shellfish Immunol. 45 (2015) 680–688. doi:10.1016/j.fsi.2015.05.020.
[189] K.B. Wicher, E. Fries, Haptoglobin, a hemoglobin-binding plasma protein, is present in bony fish and mammals but not in frog and chicken, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 4168–4173. doi:10.1073/pnas.0508723103.
[190] J.R. Delanghe, M.R. Langlois, Hemopexin: a review of biological aspects and the role in laboratory medicine, Clin. Chim. Acta. 312 (2001) 13–23. doi:10.1016/S0009-8981(01)00586-1.
[191] E.E. Johnson, M. Wessling-Resnick, Iron metabolism and the innate immune response to infection, Microbes Infect. 14 (2012) 207–216. doi:10.1016/j.micinf.2011.10.001.
[192] K. Kikuchi, M. Yamashita, S. Watabe, K. Aida, The warm temperature acclimation-related 65-kDa protein, Wap65, in goldfish and its gene expression, J. Biol. Chem. 270 (1995) 17087–17092.
[193] G. Weiss, L.T. Goodnough, Anemia of Chronic Disease, N. Engl. J. Med. 352 (2005) 1011–1023. doi:10.1056/NEJMra041809.
[194] S.T. Ong, J.Z. Shan Ho, B. Ho, J.L. Ding, Iron-withholding strategy in innate immunity, Immunobiology. 211 (2006) 295–314. doi:10.1016/j.imbio.2006.02.004.
[195] P.M. Harrison, P. Arosio, The ferritins: molecular properties, iron storage function and cellular regulation, Biochim. Biophys. Acta. 1275 (1996) 161–203.
[196] C. Moore, M. Ormseth, H. Fuchs, Causes and significance of markedly elevated serum ferritin levels in an academic medical center, J. Clin. Rheumatol. Pract. Rep. Rheum. Musculoskelet. Dis. 19 (2013) 324–328. doi:10.1097/RHU.0b013e31829ce01f.

[197] H. Liu, T. Takano, E. Peatman, J. Abernathy, S. Wang, Z. Sha, et al., Molecular characterization and gene expression of the channel catfish ferritin H subunit after bacterial infection and iron treatment, J. Exp. Zool. Part Ecol. Genet. Physiol. 313A (2010) 359–368. doi:10.1002/jez.606.
[198] Y. Hu, W. Zheng, L. Sun, Identification and molecular analysis of a ferritin subunit from red drum (*Sciaenops ocellatus*), Fish Shellfish Immunol. 28 (2010) 678–686. doi:10.1016/j.fsi.2010.01.001.
[199] T. Ganz, E. Nemeth, Hepcidin and disorders of iron metabolism, Annu. Rev. Med. 62 (2011) 347–360. doi:10.1146/annurev-med-050109-142444.
[200] S.A.M. Martin, S.C. Blaney, D.F. Houlihan, C.J. Secombes, Transcriptome response following administration of a live bacterial vaccine in Atlantic salmon (*Salmo salar*), Mol. Immunol. 43 (2006) 1900–1911. doi:10.1016/j.molimm.2005.10.007.
[201] P.N.S. Rodrigues, S. Vázquez-Dorado, J.V. Neves, J.M. Wilson, Dual function of fish hepcidin: Response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*), Dev. Comp. Immunol. 30 (2006) 1156–1167. doi:10.1016/j.dci.2006.02.005.
[202] H. Shike, X. Lauth, M.E. Westerman, V.E. Ostland, J.M. Carlberg, J.C. Van Olst, et al., Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge, Eur. J. Biochem. FEBS. 269 (2002) 2232–2237.
[203] C.-K. Khoo, A.M. Abdul-Murad, B.-C. Kua, A. Mohd-Adnan, *Cryptocaryon irritans* infection induces the acute phase response in *Lates calcarifer*: A transcriptomic perspective, Fish Shellfish Immunol. 33 (2012) 788–794. doi:10.1016/j.fsi.2012.07.005.
[204] J. Travis, B.H. Shieh, J. Potempa, The functional role of acute phase plasma proteinase inhibitors, Tokai J. Exp. Clin. Med. 13 (1988) 313–320.
[205] J.K. Stoller, L.S. Aboussouan, α1-antitrypsin deficiency, The Lancet. 365 (2005) 2225–2236. doi:10.1016/S0140-6736(05)66781-5.
[206] S.M. Janciauskiene, R. Bals, R. Koczulla, C. Vogelmeier, T. Köhnlein, T. Welte, The discovery of α1-antitrypsin and its role in health and disease, Respir. Med. 105 (2011) 1129–1139. doi:10.1016/j.rmed.2011.02.002.
[207] J.R. Forney, S. Yang, M.C. Healey, Interaction of the human serine protease inhibitor alpha-1-antitrypsin with *Cryptosporidium parvum*, J. Parasitol. 82 (1996) 496–502.
[208] B. Magnadottir, S.S. Audunsdottir, B.T. Bragason, B. Gisladottir, Z.O. Jonsson, S. Gudmundsdottir, The acute phase response of Atlantic cod (*Gadus morhua*): humoral and cellular response, Fish Shellfish Immunol. 30 (2011) 1124–1130. doi:10.1016/j.fsi.2011.02.010.
[209] A.T. Talbot, T.G. Pottinger, T.J. Smith, M.T. Cairns, Acute phase gene expression in rainbow trout (*Oncorhynchus mykiss*) after exposure to a confinement stressor: A comparison of pooled and individual data, Fish Shellfish Immunol. 27 (2009) 309–317. doi:10.1016/j.fsi.2009.05.016.
[210] J.N. Lü, J. Chen, X.J. Lu, Y.H. Shi, Identification of α1-antitrypsin as a positive acute phase protein in ayu (*Plecoglossus altivelis*) associated with *Listonella anguillarum* infection, Fish Shellfish Immunol. 32 (2012) 237–241. doi:10.1016/j.fsi.2011.11.002.

[211] L. Kilpatrick, L. McCawley, V. Nachiappan, W. Greer, S. Majumdar, H.M. Korchak, et al., Alpha-1-antichymotrypsin inhibits the NADPH oxidase-enzyme complex in phorbol ester-stimulated neutrophil membranes, J. Immunol. Baltim. Md 1950. 149 (1992) 3059–3065.
[212] P.B. Armstrong, Role of α2-macroglobulin in the immune responses of invertebrates, Invertebrate Survival Journal. 7 (2010) 165–180.
[213] M. Pushpanathan, P. Gunasekaran, J. Rajendhran, Antimicrobial peptides: versatile biological properties, Int. J. Pept. 2013 (2013) e675391. doi:10.1155/2013/675391.
[214] P.B. Armstrong, J.P. Quigley, Alpha2-macroglobulin: an evolutionarily conserved arm of the innate immune system, Dev. Comp. Immunol. 23 (1999) 375–390.
[215] M. Nonaka, A. Kimura, Genomic view of the evolution of the complement system, Immunogenetics. 58 (2006) 701–713. doi:10.1007/s00251-006-0142-1.
[216] I.E. Samonte, A. Sato, W.E. Mayer, S. Shintani, J. Klein, Linkage relationships of genes coding for α2-Macroglobulin, C3 and C4 in the zebrafish: Implications for the evolution of the complement and Mhc systems, Scand. J. Immunol. 56 (2002) 344–352. doi:10.1046/j.1365-3083.2002.01154.x.
[217] S.J. Freedman, The role of alpha 2-macroglobulin in furunculosis: a comparison of rainbow trout and brook trout, Comp. Biochem. Physiol. B. 98 (1991) 549–553.
[218] J. Mutsuro, M. Nakao, K. Fujiki, T. Yano, Multiple forms of alpha2-macroglobulin from a bony fish, the common carp (*Cyprinus carpio*): striking sequence diversity in functional sites, Immunogenetics. 51 (2000) 847–855.
[219] F.L. Li, C.P. Lu, Purification and characterization of α2-macroglobulin from grass carp *Ctenopharyngodon idellus*: Cloning a segment of the corresponding gene, Fish Shellfish Immunol. 20 (2006) 474–481. doi:10.1016/j.fsi.2005.06.006.
[220] M.X. Chang, P. Nie, G.Y. Liu, Y. Song, Q. Gao, Identification of immune genes in grass carp Ctenopharyngodon idella in response to infection of the parasitic copepod Sinergasilus major, Parasitol. Res. 96 (2005) 224–229. doi:10.1007/s00436-005-1340-8.
[221] A.E. Ellis, Inhibition of the *Aeromonas salmonicida* extracellular protease by α2-macroglobulin in the serum of rainbow trout, Microb. Pathog. 3 (1987) 167–177. doi:10.1016/0882-4010(87)90093-3.
[222] P.T.K. Woo, Cryptobiosis and its control in North American fishes, Int. J. Parasitol. 31 (2001) 566–574. doi:10.1016/S0020-7519(01)00169-2.
[223] D. Davalos, K. Akassoglou, Fibrinogen as a key regulator of inflammation in disease, Semin. Immunopathol. 34 (2012) 43–62. doi:10.1007/s00281-011-0290-8.
[224] S. Kamath, G.Y.H. Lip, Fibrinogen: biochemistry, epidemiology and determinants, QJM. 96 (2003) 711–729. doi:10.1093/qjmed/hcg129.
[225] F.J. Xie, Z.P. Zhang, P. Lin, S.H. Wang, Z.H. Zou, Y.L. Wang, Identification of immune responsible fibrinogen beta chain in the liver of large yellow croaker using a modified annealing control primer system, Fish Shellfish Immunol. 27 (2009) 202–209. doi:10.1016/j.fsi.2009.04.002.
[226] F. Berri, G.F. Rimmelzwaan, M. Hanss, E. Albina, M.-L. Foucault-Grunenwald, V.B. Lê, et al., Plasminogen controls inflammation and pathogenesis of Influenza virus infections via fibrinolysis, PLoS Pathog. 9 (2013) e1003229. doi:10.1371/journal.ppat.1003229.

[227] R.H. Easy, N.W. Ross, Changes in Atlantic salmon (Salmo salar) epidermal mucus protein composition profiles following infection with sea lice (Lepeophtheirus salmonis), Comp. Biochem. Physiol. Part D Genomics Proteomics. 4 (2009) 159–167. doi:10.1016/j.cbd.2009.02.001.
[228] S. Zhang, P. Cui, Complement system in zebrafish, Dev. Comp. Immunol. 46 (2014) 3–10. doi:10.1016/j.dci.2014.01.010.
[229] M.M. Markiewski, J.D. Lambris, The role of complement in inflammatory diseases from behind the scenes into the spotlight, Am. J. Pathol. 171 (2007) 715–727. doi:10.2353/ajpath.2007.070166.
[230] Y. Endo, M. Matsushita, T. Fujita, The role of ficolins in the lectin pathway of innate immunity, Int. J. Biochem. Cell Biol. 43 (2011) 705–712. doi:10.1016/j.biocel.2011.02.003.
[231] J.R. Dunkelberger, W.-C. Song, Complement and its role in innate and adaptive immune responses, Cell Res. 20 (2009) 34–50. doi:10.1038/cr.2009.139.
[232] H. Rus, C. Cudrici, F. Niculescu, The role of the complement system in innate immunity, Immunol. Res. 33 (2005) 103–112. doi:10.1385/IR:33:2:103.
[233] N.S. Merle, S.E. Church, V. Fremeaux-Bacchi, L.T. Roumenina, Complement system part I – molecular mechanisms of activation and regulation, Mol. Innate Immun. (2015) 262. doi:10.3389/fimmu.2015.00262.
[234] Q. Peng, K. Li, S.H. Sacks, W. Zhou, The role of anaphylatoxins C3a and C5a in regulating innate and adaptive immune responses, Inflamm. Allergy Drug Targets. 8 (2009) 236–246.
[235] M. Nonaka, S.L. Smith, Complement system of bony and cartilaginous fish, Fish Shellfish Immunol. 10 (2000) 215–228. doi:10.1006/fsim.1999.0252.
[236] J.O. Sunyer, J.D. Lambris, Evolution and diversity of the complement system of poikilothermic vertebrates, Immunol. Rev. 166 (1998) 39–57.
[237] T. Fujita, M. Matsushita, Y. Endo, The lectin-complement pathway – its role in innate immunity and evolution, Immunol. Rev. 198 (2004) 185–202. doi:10.1111/j.0105-2896.2004.0123.x.
[238] M. Nakao, J. Mutsuro, M. Nakahara, Y. Kato, T. Yano, Expansion of genes encoding complement components in bony fish: biological implications of the complement diversity, Dev. Comp. Immunol. 27 (2003) 749–762.
[239] M. Nakao, T. Kajiya, Y. Sato, T. Somamoto, Y. Kato-Unoki, M. Matsushita, et al., Lectin pathway of bony fish complement: identification of two homologs of the mannose-binding lectin associated with MASP2 in the common carp (*Cyprinus carpio*), J. Immunol. 177 (2006) 5471–5479. doi:10.4049/jimmunol.177.8.5471.
[240] H. Boshra, J. Li, J.O. Sunyer, Recent advances on the complement system of teleost fish, Fish Shellfish Immunol. 20 (2006) 239–262. doi:10.1016/j.fsi.2005.04.004.
[241] G. Forn-Cuní, E.S. Reis, S. Dios, D. Posada, J.D. Lambris, A. Figueras, et al., The evolution and appearance of C3 duplications in fish originate an exclusive teleost c3 gene form with anti-inflammatory activity, PloS One. 9 (2014) e99673. doi:10.1371/journal.pone.0099673.

[242] M. Nakao, J. Mutsuro, R. Obo, K. Fujiki, M. Nonaka, T. Yano, Molecular cloning and protein analysis of divergent forms of the complement component C3 from a bony fish, the common carp (*Cyprinus carpio*): presence of variants lacking the catalytic histidine, Eur. J. Immunol. 30 (2000) 858–866. doi:10.1002/1521-4141(200003)30:3<858::AID-IMMU858>3.0.CO;2-M.
[243] H. Boshra, J. Li, R. Peters, J. Hansen, A. Matlapudi, J.O. Sunyer, Cloning, expression, cellular distribution, and role in chemotaxis of a C5a receptor in rainbow trout: the first identification of a C5a receptor in a nonmammalian species, J. Immunol. Baltim. Md 1950. 172 (2004) 4381–4390.
[244] J.O. Sunyer, H. Boshra, J. Li, Evolution of anaphylatoxins, their diversity and novel roles in innate immunity: insights from the study of fish complement, Vet. Immunol. Immunopathol. 108 (2005) 77–89. doi:10.1016/j.vetimm.2005.07.009.
[245] T. Fournier, N. Medjoubi-N, D. Porquet, Alpha-1-acid glycoprotein, Biochim. Biophys. Acta BBA - Protein Struct. Mol. Enzymol. 1482 (2000) 157–171. doi:10.1016/S0167-4838(00)00153-9.
[246] Z.H. Israili, P.G. Dayton, Human alpha-1-glycoprotein and its interactions with drugs, Drug Metab. Rev. 33 (2001) 161–235. doi:10.1081/DMR-100104402.
[247] T. Hochepied, F.G. Berger, H. Baumann, C. Libert, Alpha(1)-acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties, Cytokine Growth Factor Rev. 14 (2003) 25–34.
[248] O. Nakamura, Y. Nozawa, E. Saito, D. Ikeda, S. Tsutsui, An alpha-1-acid glycoprotein-like protein as a major component of the ovarian cavity fluid of viviparous fish, *Neoditrema ransonnetii* (Perciformes, Embiotocidae), Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 153 (2009) 222–229. doi:10.1016/j.cbpa.2009.02.018.
[249] Y. Oba, Y. Shimasaki, Y. Oshima, H. Satone, T. Kitano, M. Nakao, et al., Purification and characterization of tributyltin-binding protein type 2 from plasma of Japanese flounder, *Paralichthys olivaceus*, J. Biochem. (Tokyo). 142 (2007) 229–238. doi:10.1093/jb/mvm119.
[250] R.S. Jack, X. Fan, M. Bernheiden, G. Rune, M. Ehlers, A. Weber, et al., Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection, Nature. 389 (1997) 742–745. doi:10.1038/39622.
[251] N. Lamping, A. Hoess, B. Yu, T.C. Park, C.J. Kirschning, D. Pfeil, et al., Effects of site-directed mutagenesis of basic residues (Arg 94, Lys 95, Lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation, J. Immunol. Baltim. Md 1950. 157 (1996) 4648–4656.
[252] N. Lamping, R. Dettmer, N.W. Schröder, D. Pfeil, W. Hallatschek, R. Burger, et al., LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria, J. Clin. Invest. 101 (1998) 2065–2071. doi:10.1172/JCI2338.
[253] J.-W. Kim, L. Gerwick, C.-I. Park, Molecular identification and expression analysis of two distinct BPI/LBPs (bactericidal permeability-increasing protein/LPS-binding protein) from rock bream, *Oplegnathus fasciatus*, Fish Shellfish Immunol. 33 (2012) 75–84. doi:10.1016/j.fsi.2012.04.003.

[254] H. Inagawa, T. Honda, C. Kohchi, T. Nishizawa, Y. Yoshiura, T. Nakanishi, et al., Cloning and characterization of the homolog of mammalian lipopolysaccharide-binding protein and bactericidal permeability-increasing protein in rainbow trout *Oncorhynchus mykiss*, J. Immunol. Baltim. Md 1950. 168 (2002) 5638–5644.
[255] J. Stenvik, T. Solstad, C. Strand, I. Leiros, T.Ø. Jørgensen T, Cloning and analyses of a BPI/LBP cDNA of the Atlantic cod (*Gadus morhua* L.), Dev. Comp. Immunol. 28 (2004) 307–323.
[256] G.H. Fu, F. Liu, J.H. Xia, G.H. Yue, The LBP gene and its association with resistance to *Aeromonas hydrophila* in tilapia, Int. J. Mol. Sci. 15 (2014) 22028–22041. doi:10.3390/ijms151222028.
[257] A. Oladiran, Studies on the mechanims of immune evasion in *Trypanosoma carassii* infections of the goldfish (*Carassius auratus* L.), Doctoral Dissertation, University of Alberta, 2012.
[258] WHO, Human African trypanosomiasis (sleeping sickness): an epidemiological update (Revised in May 2015), Wkly Epidemiol Rec. 81 (2006) 69–80.
[259] WHO, Chagas disease in Latin America: an epidemiological update based on 2010 estimates, Wkly Epidemiol Rec. 90 (2015) 33–44.
[260] WHO, Chagas disease (American trypanosomiasis) fact sheet (Revised in March 2015), Wkly Epidemiol Rec. 85 (2010) 329–336.
[261] P.T. Cantey, S.L. Stramer, R.L. Townsend, H. Kamel, K. Ofafa, C.W. Todd, et al., The United States Trypanosoma cruzi Infection Study: evidence for vector-borne transmission of the parasite that causes Chagas disease among United States blood donors, Transfusion (Paris). 52 (2012) 1922–1930. doi:10.1111/j.1537-2995.2012.03581.x.
[262] S.F. O'Brien, V. Scalia, M. Goldman, W. Fan, Q.-L. Yi, I.R. Dines, et al., Selective testing for Trypanosoma cruzi: the first year after implementation at Canadian Blood Services, Transfusion (Paris). 53 (2013) 1706–1713. doi:10.1111/j.1537-2995.2012.03950.x.
[263] J.D. Stanaway, G. Roth, The Burden of Chagas Disease: Estimates and Challenges, Glob. Heart. 10 (2015) 139–144. doi:10.1016/j.gheart.2015.06.001.
[264] L.O. Andrade, N.W. Andrews, The *Trypanosoma cruzi*-host-cell interplay: location, invasion, retention, Nat. Rev. Microbiol. 3 (2005) 819–823. doi:10.1038/nrmicro1249.
[265] A. Flávia Nardy, C.G. Freire-de-Lima, A. Morrot, Immune evasion strategies of *Trypanosoma cruzi*, J. Immunol. Res. 2015 (2015). doi:10.1155/2015/178947.
[266] C. Bogdan, M. Röllinghoff, How do protozoan parasites survive inside macrophages?, Parasitol. Today Pers. Ed. 15 (1999) 22–28.
[267] F.R.S. Gutierrez, P.M.M. Guedes, R.T. Gazzinelli, J.S. Silva, The role of parasite persistence in pathogenesis of Chagas heart disease, Parasite Immunol. 31 (2009) 673–685. doi:10.1111/j.1365-3024.2009.01108.x.
[268] F. Nagajyothi, F.S. Machado, B.A. Burleigh, L.A. Jelicks, P.E. Scherer, S. Mukherjee, et al., Mechanisms of *Trypanosoma cruzi* persistence in Chagas disease, Cell. Microbiol. 14 (2012) 634–643. doi:10.1111/j.1462-5822.2012.01764.x.

[269] Z. Brener, R.T. Gazzinelli, Immnunological control of *Trypanosoma cruzi* infection and pathogenesis of Chagas Disease, Int. Arch. Allergy Immunol. 114 (1997) 103–110. doi:10.1159/000237653.
[270] K.M. Bonney, D.M. Engman, Chagas heart disease pathogenesis: One mechanism or many?, Curr. Mol. Med. 8 (2008) 510–518.
[271] L.J. Morrison, Parasite-driven pathogenesis in *Trypanosoma brucei* infections, Parasite Immunol. 33 (2011) 448–455. doi:10.1111/j.1365-3024.2011.01286.x.
[272] A. Oladiran, M. Belosevic, Immune evasion strategies of trypanosomes: A review, J. Parasitol. 98 (2012) 284–292. doi:10.1645/GE-2925.1.
[273] W.I. Morrison, M. Murray, P.D. Sayer, J.M. Preston, The pathogenesis of experimentally induced *Trypanosoma brucei* infection in the dog. I. Tissue and organ damage., Am. J. Pathol. 102 (1981) 168–181.
[274] B. Namangala, How the African trypanosomes evade host immune killing, Parasite Immunol. 33 (2011) 430–437. doi:10.1111/j.1365-3024.2011.01280.x.
[275] D. Sacks, A. Sher, Evasion of innate immunity by parasitic protozoa, Nat. Immunol. 3 (2002) 1041–1047. doi:10.1038/ni1102-1041.
[276] P.T.K. Woo, Fish Diseases and Disorders, CABI, 2006.
[277] A.K.M.N. Islam, P.T.K. Woo, Anemia and its mechanism in goldfish *Carassius auratus* infected with *Trypanosoma danilewskyi*., Dis. Aquat. Organ. 11 (1991) 37–43. doi:10.3354/dao011037.
[278] R. Wang, M. Belosevic, Cultivation of *Trypanosoma danilewskyi* (Laveran & Mesnil, 1904) in serum-free medium and assessment of the course of infection in goldfish, *Carassius auratus* (L.), J. Fish Dis. 17 (1994) 47–56. doi:10.1111/j.1365-2761.1994.tb00344.x.
[279] P. Overath, J. Haag, M.G. Mameza, A. Lischke, Freshwater fish trypanosomes: definition of two types, host control by antibodies and lack of antigenic variation, Parasitology. 119 (Pt 6) (1999) 591–601.
[280] P.T. Woo, Acquired immunity against *Trypanosoma danilewskyi* in goldfish, *Carassius auratus*, Parasitology. 83 (1981) 343–346.
[281] D.R. Bienek, D.A. Plouffe, G.F. Wiegertjes, M. Belosevic, Immunization of goldfish with excretory/secretory molecules of *Trypanosoma danilewskyi* confers protection against infection, Dev. Comp. Immunol. 26 (2002) 649–657.
[282] D.A. Plouffe, M. Belosevic, Antibodies that recognize alpha- and beta-tubulin inhibit in vitro growth of the fish parasite *Trypanosoma danilewskyi*, Laveran and Mesnil, 1904, Dev. Comp. Immunol. 30 (2006) 685–697. doi:10.1016/j.dci.2005.09.006.
[283] B.A. Katzenback, D.A. Plouffe, G. Haddad, M. Belosevic, Administration of recombinant parasite β-tubulin to goldfish (*Carassius auratus* L.) confers partial protection against challenge infection with *Trypanosoma danilewskyi* Laveran and Mesnil, 1904, Vet. Parasitol. 151 (2008) 36–45. doi:10.1016/j.vetpar.2007.09.030.
[284] J. Charles A Janeway, P. Travers, M. Walport, M.J. Shlomchik, Immunological memory, (2001). http://www.ncbi.nlm.nih.gov/books/NBK27158/ (accessed October 6, 2015).
[285] J. Ye, I.M. Kaattari, C. Ma, S. Kaattari, The teleost humoral immune response, Fish Shellfish Immunol. 35 (2013) 1719–1728.

[286] C.H.J. Lamers, M.J.H. de HAAS, W.B. van MUISWINKEL, The reaction of the immune system of fish to vaccination: development of immunological memory in carp, Cyprinus carpio L., following direct immersion in Aeromonas hydrophila bacterin, J. Fish Dis. 8 (1985) 253–262. doi:10.1111/j.1365-2761.1985.tb00941.x.

[287] I. Dykova, J. Lom, Histopathological changes in *Trypanosoma danilewskyi* Laveran & Mesnil, 1904 and *Trypanoplasma borelli* Laveran & Mesnil, 1902 infections of goldfish, *Carassius auratus* (L)., J. Fish Dis. 2 (1979) 381–390. doi:10.1111/j.1365-2761.1979.tb00390.x.

[288] D.R. Bienek, The Biologicai and Immunological Aspect of the Host-Parasite Relationship of Goldfish, *Carassius auratus*, lnfected with *Trypanosorna danilewskyi*, Doctoral Dissertation, University of Alberta, 2001.

[289] B.A. Katzenback, D.A. Plouffe, M. Belosevic, Goldfish (*Carassius auratus* L.) possess natural antibodies with trypanocidal activity towards Trypanosoma carassii in vitro, Fish Shellfish Immunol. 34 (2013) 1025–1032. doi:10.1016/j.fsi.2012.12.018.

[290] G.E. Kaiko, J.C. Horvat, K.W. Beagley, P.M. Hansbro, Immunological decision-making: how does the immune system decide to mount a helper T-cell response?, Immunology. 123 (2008) 326–338. doi:10.1111/j.1365-2567.2007.02719.x.

[291] S. Romagnani, T-cell subsets (Th1 versus Th2), Ann. Allergy Asthma Immunol. Off. Publ. Am. Coll. Allergy Asthma Immunol. 85 (2000) 9–18. doi:10.1016/S1081-1206(10)62426-X.

[292] D. McMahon-Pratt, J. Alexander, Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease?, Immunol. Rev. 201 (2004) 206–224. doi:10.1111/j.0105-2896.2004.00190.x.

[293] Y. Belkaid, K.F. Hoffmann, S. Mendez, S. Kamhawi, M.C. Udey, T.A. Wynn, et al., The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti–IL-10 receptor antibody for sterile cure, J. Exp. Med. 194 (2001) 1497–1506.

[294] J.S. Silva, P.J. Morrissey, K.H. Grabstein, K.M. Mohler, D. Anderson, S.G. Reed, Interleukin 10 and interferon gamma regulation of experimental *Trypanosoma cruzi* infection, J. Exp. Med. 175 (1992) 169–174.

[295] F. Cardillo, J.C. Voltarelli, S.G. Reed, J.S. Silva, Regulation of *Trypanosoma cruzi* infection in mice by gamma interferon and interleukin 10: role of NK cells, Infect. Immun. 64 (1996) 128–134.

[296] J.S. Silva, D.R. Twardzik, S.G. Reed, Regulation of *Trypanosoma cruzi* infections in vitro and in vivo by transforming growth factor beta (TGF-beta), J. Exp. Med. 174 (1991) 539–545.

[297] M.A. Zacks, J.-J. Wen, G. Vyatkina, V. Bhatia, N. Garg, An overview of chagasic cardiomyopathy: pathogenic importance of oxidative stress, An. Acad. Bras. Ciênc. 77 (2005) 695–715. doi:10.1590/S0001-37652005000400009.

[298] M.I. Antúnez, R.L. Cardoni, IL-12 and IFN-γ production, and NK cell activity, in acute and chronic experimental *Trypanosoma cruzi* infections, Immunol. Lett. 71 (2000) 103–109. doi:10.1016/S0165-2478(99)00172-8.

[299] J.S. Silva, G.N. Vespa, M.A. Cardoso, J.C. Aliberti, F.Q. Cunha, Tumor necrosis factor alpha mediates resistance to *Trypanosoma cruzi* infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages., Infect. Immun. 63 (1995) 4862–4867.
[300] N. Inoue, M. Inoue, K. Kuriki, H. Yamaguchi, H. Nagasawa, T. Mikami, et al., Interleukin 4 is a crucial cytokine in controlling *Trypanosoma brucei gambiense* infection in mice, Vet. Parasitol. 86 (1999) 173–184. doi:10.1016/S0304-4017(99)00143-0.
[301] M. Hamadien, N. Lycke, M. Bakhiet, Induction of the trypanosome lymphocyte-triggering factor (TLTF) and neutralizing antibodies to the TLTF in experimental african trypanosomiasis, Immunology. 96 (1999) 606–611.
[302] A. Darji, A. Beschin, M. Sileghem, H. Heremans, L. Brys, P. De Baetselier, In vitro simulation of immunosuppression caused by *Trypanosoma brucei*: active involvement of gamma interferon and tumor necrosis factor in the pathway of suppression, Infect. Immun. 64 (1996) 1937–1943.
[303] A. Oladiran, D. Beauparlant, M. Belosevic, The expression analysis of inflammatory and antimicrobial genes in the goldfish (*Carassius auratus* L.) infected with *Trypanosoma carassii*, Fish Shellfish Immunol. 31 (2011) 606–613. doi:10.1016/j.fsi.2011.07.008.
[304] C.M.S. Ribeiro, M.J.S.L. Pontes, S. Bird, M. Chadzinska, M. Scheer, B.M.L. Verburg-van Kemenade, et al., Trypanosomiasis-induced Th17-like immune responses in carp, PLoS ONE. 5 (2010) e13012. doi:10.1371/journal.pone.0013012.
[305] A. Oladiran, M. Belosevic, *Trypanosoma carassii* hsp70 increases expression of inflammatory cytokines and chemokines in macrophages of the goldfish (*Carassius auratus* L.), Dev. Comp. Immunol. 33 (2009) 1128–1136. doi:10.1016/j.dci.2009.06.003.
[306] A. Lischke, C. Klein, Y.D. Stierhof, M. Hempel, A. Mehlert, I.C. Almeida, et al., Isolation and characterization of glycosylphosphatidylinositol-anchored, mucin-like surface glycoproteins from bloodstream forms of the freshwater-fish parasite *Trypanosoma carassii*., Biochem. J. 345 (2000) 693–700.
[307] V.L. Pereira-Chioccola, A. Acosta-Serrano, I.C. de Almeida, M.A. Ferguson, T. Souto-Padron, M.M. Rodrigues, et al., Mucin-like molecules form a negatively charged coat that protects *Trypanosoma cruzi* trypomastigotes from killing by human anti-alpha-galactosyl antibodies, J. Cell Sci. 113 (2000) 1299–1307.
[308] D.V. Devine, R.J. Falk, A.E. Balber, Restriction of the alternative pathway of human complement by intact *Trypanosoma brucei* subsp. *gambiense*., Infect. Immun. 52 (1986) 223–229.
[309] A. Ferrante, A. c. Allison, Alternative pathway activation of complement by African trypanosomes lacking a glycoprotein coat, Parasite Immunol. 5 (1983) 491–498. doi:10.1111/j.1365-3024.1983.tb00763.x.
[310] J.E. Taylor, G. Rudenko, Switching trypanosome coats: what's in the wardrobe?, Trends Genet. TIG. 22 (2006) 614–620. doi:10.1016/j.tig.2006.08.003.
[311] D.J. LaCount, A.E. Gruszynski, P.M. Grandgenett, J.D. Bangs, J.E. Donelson, Expression and function of the *Trypanosoma brucei* major surface protease (GP63) genes, J. Biol. Chem. 278 (2003) 24658–24664. doi:10.1074/jbc.M301451200.

[312] A. Oladiran, M. Belosevic, Recombinant glycoprotein 63 (Gp63) of *Trypanosoma carassii* suppresses antimicrobial responses of goldfish (*Carassius auratus* L.) monocytes and macrophages, Int. J. Parasitol. 42 (2012) 621–633. doi:10.1016/j.ijpara.2012.04.012.

[313] D.A. Plouffe, M. Belosevic, Enzyme treatment of *Trypanosoma danilewskyi* (Laveran & Mesnil) increases its susceptibility to lysis by the alternative complement pathway of goldfish, *Carassius auratus* (L.), J. Fish Dis. 27 (2004) 277–285. doi:10.1111/j.1365-2761.2004.00541.x.

[314] V. Ferreira, C. Valck, G. Sánchez, A. Gingras, S. Tzima, M.C. Molina, et al., The classical activation pathway of the human complement system is specifically inhibited by calreticulin from *Trypanosoma cruzi*, J. Immunol. 172 (2004) 3042–3050. doi:10.4049/jimmunol.172.5.3042.

[315] G. Kasper, A. Brown, M. Eberl, L. Vallar, N. Kieffer, C. Berry, et al., A calreticulin-like molecule from the human hookworm Necator americanus interacts with C1q and the cytoplasmic signalling domains of some integrins, Parasite Immunol. 23 (2001) 141–152. doi:10.1046/j.1365-3024.2001.00366.x.

[316] V. Ferreira, M.C. Molina, W. Schwaeble, D. Lemus, A. Ferreira, Does *Trypanosoma cruzi* calreticulin modulate the complement system and angiogenesis?, Trends Parasitol. 21 (2005) 169–174. doi:10.1016/j.pt.2005.02.005.

[317] R.M. Ngure, P. David Eckersall, F.W. Jennings, J.M. Burke, M.J. Stear, P.G.E. Kennedy, et al., Major acute phase response of haptoglobin and serum amyloid-P following experimental infection of mice with *Trypanosoma brucei brucei*, Parasitol. Int. 46 (1997) 247–254. doi:10.1016/S1383-5769(97)00034-2.

[318] M.M. Costa, S.T. Dos Anjos Lopes, R.T. França, A.S. da Silva, F.C. Paim, H.E. Palma, et al., Role of acute phase proteins in the immune response of rabbits infected with *Trypanosoma evansi*, Res. Vet. Sci. 95 (2013) 182–188. doi:10.1016/j.rvsc.2013.01.029.

[319] F.A. Lara, C. Sant'anna, D. Lemos, G. a. T. Laranja, M.G.P. Coelho, I. Reis Salles, et al., Heme requirement and intracellular trafficking in *Trypanosoma cruzi* epimastigotes, Biochem. Biophys. Res. Commun. 355 (2007) 16–22. doi:10.1016/j.bbrc.2006.12.238.

[320] B. Vanhollebeke, G.D. Muylder, M.J. Nielsen, A. Pays, P. Tebabi, M. Dieu, et al., A haptoglobin-hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans, Science. 320 (2008) 677–681. doi:10.1126/science.1156296.

[321] M.K. Higgins, O. Tkachenko, A. Brown, J. Reed, J. Raper, M. Carrington, Structure of the trypanosome haptoglobin–hemoglobin receptor and implications for nutrient uptake and innate immunity, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 1905–1910. doi:10.1073/pnas.1214943110.

[322] P. Borst, A.H. Fairlamb, Surface receptors and transporters of *Trypanosoma Brucei*, Annu. Rev. Microbiol. 52 (1998) 745–778. doi:10.1146/annurev.micro.52.1.745.

[323] A.B. Smith, S.L. Hajduk, Identification of haptoglobin as a natural inhibitor of trypanocidal activity in human serum, Proc. Natl. Acad. Sci. 92 (1995) 10262–10266.

[324] M. Fernández, M. Fernández, The role of haptoglobin genotypes in Chagas Disease, Dis. Markers Dis. Markers. 2014, 2014 (2014) e793646. doi:10.1155/2014/793646, 10.1155/2014/793646.

[325] D.R. Calderoni, T.D.S. Andrade, H.Z.W. Grotto, Haptoglobin phenotype appears to affect the pathogenesis of American trypanosomiasis, Ann. Trop. Med. Parasitol. 100 (2006) 213–221. doi:10.1179/136485906X86356.
[326] N. Mundaray Fernández, M. Fernández-Mestre, The role of haptoglobin genotypes in Chagas disease, Dis. Markers. 2014 (2014) 793646. doi:10.1155/2014/793646.
[327] D. Schell, N.K. Borowy, P. Overath, Transferrin is a growth factor for the bloodstream form of *Trypanosoma brucei*, Parasitol. Res. 77 (1991) 558–560.
[328] H.G.A.M. van Luenen, R. Kieft, R. Mussmann, M. Engstler, B. ter Riet, P. Borst, Trypanosomes change their transferrin receptor expression to allow effective uptake of host transferrin, Mol. Microbiol. 58 (2005) 151–165. doi:10.1111/j.1365-2958.2005.04831.x.
[329] W. Bitter, H. Gerrits, R. Kieft, P. Borst, The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*, Nature. 391 (1998) 499–502. doi:10.1038/35166.
[330] B. Fast, K. Kremp, M. Boshart, D. Steverding, Iron-dependent regulation of transferrin receptor expression in *Trypanosoma brucei*, Biochem. J. 342 Pt 3 (1999) 691–696.
[331] B. Stijlemans, G. Caljon, S.K.A. Natesan, D. Saerens, K. Conrath, D. Pérez-Morga, et al., High affinity nanobodies against the *Trypanosome brucei* VSG are potent trypanolytic agents that block endocytosis, PLoS Pathog. 7 (2011) e1002072. doi:10.1371/journal.ppat.1002072.
[332] H. Kitani, Y. Yagi, J. Naessens, K. Sekikawa, F. Iraqi, The secretion of acute phase proteins and inflammatory cytokines during Trypanosoma congolense infection is not affected by the absence of the TNF-α gene, Acta Trop. 92 (2004) 35–42. doi:10.1016/j.actatropica.2004.05.013.
[333] B. Stijlemans, A. Vankrunkelsven, L. Brys, S. Magez, P. De Baetselier, Role of iron homeostasis in trypanosomiasis-associated anemia, Immunobiology. 213 (2008) 823–835. doi:10.1016/j.imbio.2008.07.023.
[334] A.S. Da Silva, L. Hoehne, A.A. Tonin, R.A. Zanette, P. Wolkmer, M.M. Costa, et al., Trypanosoma evansi: Levels of copper, iron and zinc in the bloodstream of infected cats, Exp. Parasitol. 123 (2009) 35–38. doi:10.1016/j.exppara.2009.05.007.
[335] M.J. Friedman, Control of malaria virulence by alpha 1-acid glycoprotein (orosomucoid), an acute-phase (inflammatory) reactant., Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 5421–5424.
[336] N. Tiberti, V. Lejon, D. Mumba Ngoyi, E. Matovu, J. Enyaru, N. Walter, et al., Increased acute immune response during the meningo-encephalitic stage of *Trypanosoma brucei rhodesiense* sleeping sickness compared to *Trypanosoma brucei gambiense*, Adv. Integr. Med. 6 (2015) 1–9. doi:10.1016/j.trprot.2014.11.001.
[337] M.E.A. Pereira, A developmentally regulated neuraminidase activity in *Trypanosoma cruzi*, Science. 219 (1983) 1444–1446.
[338] J.M. Ndung'u, P.D. Eckersall, F.W. Jennings, Elevation of the concentration of acute phase proteins in dogs infected with *Trypanosoma brucei*, Acta Trop. 49 (1991) 77–86.

[339] C.A. da Silva, A. Fattori, A.L. Sousa, S.B. Mazon, S.M. Alegre, E.A. Almeida, et al., Determining the C-Reactive protein level in patients with different clinical forms of Chagas Disease, Rev. Esp. Cardiol. Engl. Ed. 63 (2010) 1096–1099. doi:10.1016/S1885-5857(10)70215-2.
[340] M. Beucher, K.A. Norris, Sequence diversity of the *Trypanosoma cruzi* complement regulatory protein family, Infect. Immun. 76 (2008) 750–758. doi:10.1128/IAI.01104-07.
[341] I. Cestari, I. Evans-Osses, L.J. Schlapbach, I. de Messias-Reason, M.I. Ramirez, Mechanisms of complement lectin pathway activation and resistance by trypanosomatid parasites, Mol. Immunol. 53 (2013) 328–334. doi:10.1016/j.molimm.2012.08.015.
[342] S.F. Gonzalez, K. Buchmann, M.E. Nielsen, *Ichthyophthirius multifiliis* infection induces massive up-regulation of serum amyloid A in carp (*Cyprinus carpio*), Vet. Immunol. Immunopathol. 115 (2007) 172–178. doi:10.1016/j.vetimm.2006.09.007.
[343] I. Maudlin, P.H. Holmes, M.A. Miles, The Trypanosomiases, CABI, 2004.
[344] C. Valck, G. Ramírez, N. López, C.H. Ribeiro, I. Maldonado, G. Sánchez, et al., Molecular mechanisms involved in the inactivation of the first component of human complement by *Trypanosoma cruzi* calreticulin, Mol. Immunol. 47 (2010) 1516–1521. doi:10.1016/j.molimm.2010.01.019.
[345] A. Koj, Cytokines regulating acute inflammation and synthesis of acute phase proteins, Blut. 51 (1985) 267–274. doi:10.1007/BF00320521.
[346] T.C. Araujo-Jorge, M.-J.F. Lage, M.T. Rivera, Y. Carlier, F.V. Leuven, *Trypanosoma cruzi*: Enhanced alpha-macroglobulin levels correlate with the resistance of BALB/cj mice to acute infection, Parasitol. Res. 78 (1992) 215–221. doi:10.1007/BF00931729.
[347] C.M.L.M. Coutinho, G.H. Cavalcanti, F. van Leuven, T.C. Araújo-Jorge, C.M.L.M. Coutinho, Alpha-2-macroglobulin binds to the surface of *Trypanosoma cruzi*, Parasitol. Res. 83 (1997) 144–150. doi:10.1007/s004360050224.
[348] A. Ramos, M.S. Remedi, C. Sánchez, G. Bonacci, M.A. Vides, G. Chiabrando, Inhibitory effects of human alpha 2-macroglobulin on *Trypanosoma cruzi* epimastigote proteinases, Acta Trop. 68 (1997) 327–337.
[349] J. Scharfstein, Parasite cysteine proteinase interactions with α2-macroglobulin or kininogens: differential pathways modulating inflammation and innate immunity in infection by pathogenic trypanosomatids, Immunobiology. 211 (2006) 117–125. doi:10.1016/j.imbio.2005.10.014.
[350] X. Zuo, P.T. Woo, Natural anti-proteases in rainbow trout, *Oncorhynchus mykiss* and brook charr, *Salvelinus fontinalis* and the in vitro neutralization of fish alpha 2-macroglobulin by the metalloprotease from the pathogenic haemoflagellate, *Cryptobia salmositica*, Parasitology. 114 (Pt 4) (1997) 375–381.
[351] C. Gabay, I. Kushner, Acute-phase Proteins, in: eLS, John Wiley & Sons, Ltd, 2001. http://onlinelibrary.wiley.com/doi/10.1038/npg.els.0000497/abstract (accessed July 27, 2015).
[352] G.J. Anderson, D.M. Frazer, Hepatic Iron Metabolism, Semin. Liver Dis. 25 (2005) 420–432. doi:10.1055/s-2005-923314.

[353] N.E. Hellman, J.D. Gitlin, Ceruloplasmin metabolism and function, Annu. Rev. Nutr. 22 (2002) 439–458. doi:10.1146/annurev.nutr.22.012502.114457.
[354] M.W. Hentze, M.U. Muckenthaler, N.C. Andrews, Balancing acts: Molecular control of mammalian iron metabolism, Cell. 117 (2004) 285–297. doi:10.1016/S0092-8674(04)00343-5.
[355] D.R. Bienek, M. Belosevic, Comparative assessment of growth of *Trypanosoma danilewskyi* (Laveran & Mesnil) in medium containing fish or mammalian serum, J. Fish Dis. 20 (1997) 217–221. doi:10.1046/j.1365-2761.1997.00295.x.
[356] P.T. Woo, The haematocrit centrifuge for the detection of trypanosomes in blood, Can. J. Zool. 47 (1969) 921–923.
[357] N.F. Neumann, D. Barreda, M. Belosevic, Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes, Dev. Comp. Immunol. 22 (1998) 417–432. doi:10.1016/S0145-305X(98)00023-8.
[358] R. Wang, N.F. Neumann, Q. Shen, M. Belosevic, Establishment and characterization of a macrophage cell line from the goldfish, Fish Shellfish Immunol. 5 (1995) 329–346. doi:10.1006/fsim.1995.0032.
[359] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol. Biol. Evol. 28 (2011) 2731–2739. doi:10.1093/molbev/msr121.
[360] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature. 227 (1970) 680–685. doi:10.1038/227680a0.
[361] D. Hoole, J.W. Lewis, P.M.M. Schuwerack, C. Chakravarthy, A.K. Shrive, T.J. Greenhough, et al., Inflammatory interactions in fish exposed to pollutants and parasites: a role for apoptosis and C reactive protein, Parasitology. 126 (2003) S71–S85. doi:10.1017/S0031182003003779.
[362] M.R. Mauk, A. Smith, A.G. Mauk, An alternative view of the proposed alternative activities of hemopexin, Protein Sci. Publ. Protein Soc. 20 (2011) 791–805. doi:10.1002/pro.616.
[363] J. Lu, K.D. Marjon, C. Mold, T.W. Du Clos, P.D. Sun, Pentraxins and Fc receptors, Immunol. Rev. 250 (2012) 230–238. doi:10.1111/j.1600-065X.2012.01162.x.
[364] C. Cray, Acute phase proteins in animals, Prog. Mol. Biol. Transl. Sci. 105 (2012) 113–150. doi:10.1016/B978-0-12-394596-9.00005-6.
[365] C.J. Furlaneto, A. Campa, A novel function of serum amyloid A: a potent stimulus for the release of tumor necrosis factor-α, interleukin-1β, and interleukin-8 by human blood neutrophil, Biochem. Biophys. Res. Commun. 268 (2000) 405–408. doi:10.1006/bbrc.2000.2143.
[366] C. Nakahara, K. Kanemoto, N. Saito, Y. Oyake, T. Kamoda, M. Nagata, et al., C-reactive protein frequently localizes in the kidney in glomerular diseases, Clin. Nephrol. 55 (2001) 365–370.
[367] S.S. Audunsdottir, B. Magnadottir, B. Gisladottir, Z.O. Jonsson, B.T. Bragason, The acute phase response of cod (*Gadus morhua* L.): Expression of immune response genes, Fish Shellfish Immunol. 32 (2012) 360–367. doi:10.1016/j.fsi.2011.11.034.

[368] M.K. Raida, K. Buchmann, Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1, Dev. Comp. Immunol. 33 (2009) 35–45. doi:10.1016/j.dci.2008.07.001.
[369] E.M. MacCarthy, I. Burns, I. Irnazarow, A. Polwart, T.J. Greenhough, A.K. Shrive, et al., Serum CRP-like protein profile in common carp *Cyprinus carpio* challenged with *Aeromonas hydrophila* and *Escherichia coli* lipopolysaccharide, Dev. Comp. Immunol. 32 (2008) 1281–1289. doi:10.1016/j.dci.2008.04.004.
[370] S. Sinha, C. Mandal, A.K. Allen, C. Mandal, Acute phase response of C-reactive protein of *Labeo rohita* to aquatic pollutants is accompanied by the appearance of distinct molecular forms, Arch. Biochem. Biophys. 396 (2001) 139–150. doi:10.1006/abbi.2001.2592.
[371] S. Suchitra, K.A. Anbu, D.K. Rathore, M. Mahawar, B.P. Singh, P. Joshi, *Haemonchus contortus* calreticulin binds to C-reactive protein of its host, a novel survival strategy of the parasite, Parasite Immunol. 30 (2008) 371–374. doi:10.1111/j.1365-3024.2008.01028.x.
[372] U. Singh, S. Devaraj, I. Jialal, C-reactive protein decreases tissue plasminogen activator activity in human aortic endothelial cells: evidence that C-reactive protein is a procoagulant, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 2216–2221. doi:10.1161/01.ATV.0000183718.62409.ea.
[373] D. Gershov, S. Kim, N. Brot, K.B. Elkon, C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity, J. Exp. Med. 192 (2000) 1353–1364. doi:10.1084/jem.192.9.1353.
[374] A.J. Szalai, The antimicrobial activity of C-reactive protein, Microbes Infect. Inst. Pasteur. 4 (2002) 201–205.
[375] A.J. Nauta, M.R. Daha, C. van Kooten, A. Roos, Recognition and clearance of apoptotic cells: a role for complement and pentraxins, Trends Immunol. 24 (2003) 148–154.
[376] A. Agrawal, S. Bhattacharya, Possible role of C-reactive protein in detoxication of mercury, Indian J. Exp. Biol. 28 (1990) 638–641.
[377] H. Kodama, Y. Matsuoka, Y. Tanaka, Y. Liu, T. Iwasaki, S. Watarai, Changes of C-reactive protein levels in rainbow trout (*Oncorhynchus mykiss*) sera after exposure to anti-ectoparasitic chemicals used in aquaculture, Fish Shellfish Immunol. 16 (2004) 589–597. doi:10.1016/j.fsi.2003.09.007.
[378] M.B. Pepys, F.C. De Beer, C.P. Milstein, J.F. March, A. Feinstein, N. Butress, et al., C-reactive protein and serum amyloid P component in the plaice (*Pleuronectes platessa* L.), a marine teleost, are homologous with their human counterparts, Biochim. Biophys. Acta BBA - Protein Struct. Mol. Enzymol. 704 (1982) 123–133. doi:10.1016/0167-4838(82)90139-X.
[379] H. Kodama, H. Arimitsu, M. Mukamoto, C. Sugimoto, Enhancement of phagocytic and chemokinetic activities of rainbow trout head kidney cells by C-reactive protein, Am. J. Vet. Res. 60 (1999) 240–244.

[380] A. Falco, J.R. Cartwright, G.F. Wiegertjes, D. Hoole, Molecular characterization and expression analysis of two new C-reactive protein genes from common carp (*Cyprinus carpio*), Dev. Comp. Immunol. 37 (2012) 127–138. doi:10.1016/j.dci.2011.10.005.

[381] R. Chen, J. Qi, S. Yao, X. Pan, F. Gao, C. Xia, Expression, crystallization and preliminary crystallographic analysis of C-reactive protein from zebrafish, Acta Crystallograph. Sect. F Struct. Biol. Cryst. Commun. 67 (2011) 1633–1636. doi:10.1107/S1744309111037390.

[382] N. Kovacevic, M.O. Hagen, J. Xie, M. Belosevic, The analysis of the acute phase response during the course of *Trypanosoma carassii* infection in the goldfish (*Carassius auratus* L.), Dev. Comp. Immunol. 53 (2015) 112–122. doi:10.1016/j.dci.2015.06.009.

[383] L. Grayfer, M. Belosevic, Molecular characterization of tumor necrosis factor receptors 1 and 2 of the goldfish (*Carassius aurutus* L.), Mol. Immunol. 46 (2009) 2190–2199. doi:10.1016/j.molimm.2009.04.016.

[384] Q. Li, T. Kang, X. Tian, Y. Ma, M. Li, J. Richards, et al., Multimeric Stability of Human C-reactive Protein in Archived Specimens, PLoS ONE. 8 (2013). doi:10.1371/journal.pone.0058094.

[385] M. Rzychon, I. Zegers, H. Schimmel, Analysis of the physicochemical state of C-reactive protein in different preparations including 2 certified reference materials, Clin. Chem. 56 (2010) 1475–1482. doi:10.1373/clinchem.2010.147124.

[386] K.B. Bodman-Smith, M. Mbuchi, F.J. Culley, P.A. Bates, J.G. Raynes, C-reactive protein-mediated phagocytosis of *Leishmania donovani* promastigotes does not alter parasite survival or macrophage responses, Parasite Immunol. 24 (2002) 447–454. doi:10.1046/j.1365-3024.2002.00486.x.

[387] C. Lelubre, S. Anselin, K. Zouaoui Boudjeltia, P. Biston, M. Piagnerelli, l, Interpretation of C-reactive protein concentrations in critically ill patients, BioMed Res. Int. 2013 (2013) e124021. doi:10.1155/2013/124021.

[388] D.M. Vandijck, E.A. Hoste, S.I. Blot, P.O. Depuydt, R.A. Peleman, J.M. Decruyenaere, Dynamics of C-reactive protein and white blood cell count in critically ill patients with nosocomial Gram positive vs. Gram negative bacteremia: a historical cohort study, BMC Infect. Dis. 7 (2007) 106. doi:10.1186/1471-2334-7-106.

[389] N. Hurt, T. Smith, T. Teuscher, M. Tanner, Do high levels of C-reactive protein in Tanzanian children indicate malaria morbidity., Clin. Diagn. Lab. Immunol. 1 (1994) 437–444.

[390] R. Paul, P.K. Sinha, R. Bhattacharya, A.K. Banerjee, P. Raychaudhuri, J. Mondal, Study of C reactive protein as a prognostic marker in malaria from Eastern India, Adv. Biomed. Res. 1 (2012). doi:10.4103/2277-9175.100140.

[391] D.C. Borges, N.M. Araujo, C.R. Cardoso, J.E. Lazo Chica, Different parasite inocula determine the modulation of the immune response and outcome of experimental *Trypanosoma cruzi* infection, Immunology. 138 (2013) 145–156. doi:10.1111/imm.12022.

[392] P.C. Heinrich, J.V. Castell, T. Andus, Interleukin-6 and the acute phase response., Biochem. J. 265 (1990) 621–636.

[393] J.S. Yudkin, M. Kumari, S.E. Humphries, V. Mohamed-Ali, Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link?, Atherosclerosis. 148 (2000) 209–214. doi:10.1016/S0021-9150(99)00463-3.
[394] T.W. Du Clos, Pentraxins: Structure, function, and role in inflammation, ISRN Inflamm. 2013 (2013) 1–22. doi:10.1155/2013/379040.
[395] Q. Dong, J.R. Wright, Expression of C-reactive protein by alveolar macrophages, J. Immunol. Baltim. Md 1950. 156 (1996) 4815–4820.
[396] D.G. Haider, N. Leuchten, G. Schaller, G. Gouya, J. Kolodjaschna, L. Schmetterer, et al., C-reactive protein is expressed and secreted by peripheral blood mononuclear cells, Clin. Exp. Immunol. 146 (2006) 533–539. doi:10.1111/j.1365-2249.2006.03224.x.
[397] A. Nayak, L. Pednekar, K.B. Reid, U. Kishore, Complement and non-complement activating functions of C1q: A prototypical innate immune molecule, Innate Immun. 18 (2012) 350–363. doi:10.1177/1753425910396252.
[398] M. Nakao, M. Tsujikura, S. Ichiki, T.K. Vo, T. Somamoto, The complement system in teleost fish: progress of post-homolog-hunting researches, Dev. Comp. Immunol. 35 (2011) 1296–1308. doi:10.1016/j.dci.2011.03.003.
[399] A. Biro, Z. Rovo, D. Papp, L. Cervenak, L. Varga, G. Fust, et al., Studies on the interactions between C-reactive protein and complement proteins, Immunology. 121 (2007) 40–50. doi:10.1111/j.1365-2567.2007.02535.x.
[400] P.T.K. Woo, *Cryptobia (Trypanoplasma) salmositica* and salmonid cryptobiosis, J. Fish Dis. 26 (2003) 627–646.
[401] S.R.M. Jones, P.T.K. Woo, The immune response of rainbow trout, *Salmo gairdneri* Richardson, to the haemoflagellate, *Cryptobia salmositica* Katz, 1951, J. Fish Dis. 10 (1987) 395–402. doi:10.1111/j.1365-2761.1987.tb01087.x.
[402] V. Pasceri, J.T. Willerson, E.T.H. Yeh, Direct proinflammatory effect of C-reactive protein on human endothelial cells, Circulation. 102 (2000) 2165–2168. doi:10.1161/01.CIR.102.18.2165.
[403] S. Verma, C.-H. Wang, S.-H. Li, A.S. Dumont, P.W.M. Fedak, M.V. Badiwala, et al., A self-fulfilling prophecy C-reactive protein attenuates nitric oxide production and inhibits angiogenesis, Circulation. 106 (2002) 913–919. doi:10.1161/01.CIR.0000029802.88087.5E.
[404] K.E. Taylor, C.W. van den Berg, Structural and functional comparison of native pentameric, denatured monomeric and biotinylated C-reactive protein, Immunology. 120 (2007) 404–411. doi:10.1111/j.1365-2567.2006.02516.x.
[405] M. Fujita, Y.K. Takada, Y. Izumiya, Y. Takada, The binding of monomeric C-Reactive Protein (mCRP) to integrins αvβ3 and α4β1 is related to its pro-inflammatory action, PLoS ONE. 9 (2014) e93738. doi:10.1371/journal.pone.0093738.
[406] E. Hatanaka, F. Pereira Ribeiro, A. Campa, The acute phase protein serum amyloid A primes neutrophils, FEMS Immunol. Med. Microbiol. 38 (2003) 81–84. doi:10.1016/S0928-8244(03)00112-3.
[407] M.E. Gatt, S. Urieli-Shoval, L. Preciado-Patt, M. Fridkin, S. Calco, Y. Azar, et al., Effect of serum amyloid A on selected in vitro functions of isolated human neutrophils, J. Lab. Clin. Med. 132 (1998) 414–420.

[408] R.P. Linke, V. Bock, G. Valet, G. Rothe, Inhibition of the oxidative burst response of N-formyl peptide-stimulated neutrophils by serum amyloid-A protein, Biochem. Biophys. Res. Commun. 176 (1991) 1100–1105. doi:10.1016/0006-291X(91)90397-P.

[409] J. Wei, M. Guo, H. Ji, Q. Qin, Molecular cloning, characterization of one key molecule of teleost innate immunity from orange-spotted grouper (*Epinephelus coioides*): Serum amyloid A, Fish Shellfish Immunol. 34 (2013) 296–304. doi:10.1016/j.fsi.2012.11.014.

[410] K. Saranya Revathy, N. Umasuthan, I. Whang, Y. Lee, S. Lee, M.-J. Oh, et al., A novel acute phase reactant, serum amyloid A-like 1, from *Oplegnathus fasciatus*: Genomic and molecular characterization and transcriptional expression analysis, Dev. Comp. Immunol. 37 (2012) 294–305. doi:10.1016/j.dci.2012.03.014.

[411] C. Shah, R. Hari-Dass, J.G. Raynes, Serum amyloid A is an innate immune opsonin for Gram-negative bacteria, Blood. 108 (2006) 1751–1757. doi:10.1182/blood-2005-11-011932.

[412] C. Liu, Serum amyloid a protein in clinical cancer diagnosis, Pathol. Oncol. Res. POR. 18 (2012) 117–121. doi:10.1007/s12253-011-9459-7.

[413] E. Malle, F.C. De Beer, Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice, Eur. J. Clin. Invest. 26 (1996) 427–435.

[414] W.-H. Wang, L.G. McNatt, I.-H. Pang, P.E. Hellberg, J.H. Fingert, M.D. McCartney, et al., Increased expression of Serum Amyloid A in glaucoma and its effect on intraocular pressure, Investig. Opthalmology Vis. Sci. 49 (2008) 1916. doi:10.1167/iovs.07-1104.

[415] W. Li, S. Zhu, J. Li, J. D'amore, J. D'angelo, H. Yang, et al., Serum Amyloid A stimulates PKR expression and HMGB1 release possibly through TLR4/RAGE receptors, Mol. Med. Camb. Mass. (2015). doi:10.2119/molmed.2015.00109.

[416] S. Urieli-Shoval, P. Cohen, S. Eisenberg, Y. Matzner, Widespread expression of serum amyloid A in histologically normal human tissues. Predominant localization to the epithelium, J. Histochem. Cytochem. Off. J. Histochem. Soc. 46 (1998) 1377–1384.

[417] I. de Bruijn, R. Belmonte, V.L. Anderson, M. Saraiva, T. Wang, P. van West, et al., Immune gene expression in trout cell lines infected with the fish pathogenic oomycete *Saprolegnia parasitica*, Dev. Comp. Immunol. 38 (2012) 44–54. doi:10.1016/j.dci.2012.03.018.

[418] F.W. Goetz, D.B. Iliev, L.A.R. McCauley, C.Q. Liarte, L.B. Tort, J.V. Planas, et al., Analysis of genes isolated from lipopolysaccharide-stimulated rainbow trout (*Oncorhynchus mykiss*) macrophages, Mol. Immunol. 41 (2004) 1199–1210. doi:10.1016/j.molimm.2004.06.005.

[419] N. Upragarin, W.J.M. Landman, W. Gaastra, E. Gruys, Extrahepatic production of acute phase serum amyloid A, Histol. Histopathol. 20 (2005) 1295–1307.

[420] L.R. Sardinha, T. Mosca, R.M. Elias, R.S. do Nascimento, L.A. Gonçalves, D.Z. Bucci, et al., The liver plays a major role in clearance and destruction of blood trypomastigotes in *Trypanosoma cruzi* chronically infected mice, PLoS Negl. Trop. Dis. 4 (2010) e578. doi:10.1371/journal.pntd.0000578.

[421] J.L. Ather, K. Ckless, R. Martin, K.L. Foley, B.T. Suratt, J.E. Boyson, et al., Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice, J. Immunol. Baltim. Md 1950. 187 (2011) 64–73. doi:10.4049/jimmunol.1100500.

[422] N. Olsson, A. Siegbahn, G. Nilsson, Serum amyloid A induces chemotaxis of human mast cells by activating a pertussis toxin-sensitive signal transduction pathway, Biochem. Biophys. Res. Commun. 254 (1999) 143–146. doi:10.1006/bbrc.1998.9911.

[423] M. Gouwy, M. De Buck, N. Pörtner, G. Opdenakker, P. Proost, S. Struyf, et al., Serum amyloid A chemoattracts immature dendritic cells and indirectly provokes monocyte chemotaxis by induction of cooperating CC and CXC chemokines, Eur. J. Immunol. 45 (2015) 101–112. doi:10.1002/eji.201444818.

[424] R. Renckens, J.J.T.H. Roelofs, S. Knapp, A.F. de Vos, S. Florquin, T. van der Poll, The acute-phase response and serum amyloid A inhibit the inflammatory response to *Acinetobacter baumannii* Pneumonia, J. Infect. Dis. 193 (2006) 187–195. doi:10.1086/498876.

[425] L. Sun, H. Zhou, Z. Zhu, Q. Yan, L. Wang, Q. Liang, et al., Ex vivo and in vitro effect of Serum Amyloid A in the induction of macrophage M2 markers and efferocytosis of apoptotic neutrophils, J. Immunol. 194 (2015) 4891–4900. doi:10.4049/jimmunol.1402164.

[426] R. Hari-Dass, C. Shah, D.J. Meyer, J.G. Raynes, Serum amyloid A protein binds to outer membrane protein A of gram-negative bacteria, J. Biol. Chem. 280 (2005) 18562–18567. doi:10.1074/jbc.M500490200.

[427] B.L. Kagan, H. Jang, R. Capone, F. Teran Arce, S. Ramachandran, R. Lal, et al., Antimicrobial properties of amyloid peptides, Mol. Pharm. 9 (2012) 708–717. doi:10.1021/mp200419b.

[428] Y. Hirakura, I. Carreras, J.D. Sipe, B.L. Kagan, Channel formation by serum amyloid A: a potential mechanism for amyloid pathogenesis and host defense, Amyloid. 9 (2002) 13–23. doi:10.3109/13506120209072440.

[429] S.J. Soscia, J.E. Kirby, K.J. Washicosky, S.M. Tucker, M. Ingelsson, B. Hyman, et al., The Alzheimer's disease-associated amyloid β-protein Is an antimicrobial peptide, PLoS ONE. 5 (2010) e9505. doi:10.1371/journal.pone.0009505.

[430] E. Andersson, V. Rydengård, A. Sonesson, M. Mörgelin, L. Björck, A. Schmidtchen, Antimicrobial activities of heparin-binding peptides, Eur. J. Biochem. FEBS. 271 (2004) 1219–1226.

[431] E.R. Eckhardt, J. Witta, J. Zhong, R. Arsenescu, V. Arsenescu, Y. Wang, et al., Intestinal epithelial serum amyloid A modulates bacterial growth in vitro and pro-inflammatory responses in mouse experimental colitisI, BMC Gastroenterol. 10 (2010) 133. doi:10.1186/1471-230X-10-133.

[432] A. Erman, K. Lakota, K. Mrak-Poljsak, M.G. Blango, V. Krizan-Hergouth, M.A. Mulvey, et al., Uropathogenic *Escherichia coli* induces Serum Amyloid A in mice following urinary tract and systemic inoculation, PLoS ONE. 7 (2012) e32933. doi:10.1371/journal.pone.0032933.

[433] H.H. Petersen, J.P. Nielsen, P.M.H. Heegaard, Application of acute phase protein measurements in veterinary clinical chemistry, Vet. Res. 35 (2004) 163–187. doi:10.1051/vetres:2004002.

[434] T.C. Fletcher, C.J. Secombes, Immunology of Fish, in: eLS, John Wiley & Sons, Ltd, 2001. http://onlinelibrary.wiley.com/doi/10.1002/9780470015902.a0000520.pub2/abstract (accessed August 9, 2015).
[435] M.B. Pepys, P.N. Hawkins, M.C. Kahan, G.A. Tennent, J.R. Gallimore, D. Graham, et al., Proinflammatory effects of bacterial recombinant human C-reactive protein are caused by contamination with bacterial products, not by C-reactive protein itself, Circ. Res. 97 (2005) e97–e103. doi:10.1161/01.RES.0000193595.03608.08.
[436] C.W. Van Den Berg, K.E. Taylor, D. Lang, C-reactive protein-induced in vitro vasorelaxation is an artefact caused by the presence of sodium azide in commercial preparations, Arterioscler. Thromb. Vasc. Biol. 24 (2004) e168–e171. doi:10.1161/01.ATV.0000142807.92781.d9.
[437] M. Torzewski, A.B. Waqar, J. Fan, Animal models of C-reactive protein, Mediators Inflamm. 2014 (2014) 683598. doi:10.1155/2014/683598.
[438] C. Zouki, B. Haas, J.S.D. Chan, L.A. Potempa, J.G. Filep, Loss of pentameric symmetry of C-reactive protein is associated with promotion of neutrophil-endothelial cell adhesion, J. Immunol. 167 (2001) 5355–5361. doi:10.4049/jimmunol.167.9.5355.
[439] M.S. Wang, J.C. Black, M.K. Knowles, S.M. Reed, C-reactive protein (CRP) aptamer binds to monomeric but not pentameric form of CRP, Anal. Bioanal. Chem. 401 (2011) 1309–1318. doi:10.1007/s00216-011-5174-1.
[440] L. Björkman, J.G. Raynes, C. Shah, A. Karlsson, C. Dahlgren, J. Bylund, The proinflammatory activity of recombinant serum amyloid A is not shared by the endogenous protein in the circulation, Arthritis Rheum. 62 (2010) 1660–1665. doi:10.1002/art.27440.
[441] R. He, H. Sang, R.D. Ye, Serum amyloid A induces IL-8 secretion through a G protein–coupled receptor, FPRL1/LXA4R, Blood. 101 (2003) 1572–1581. doi:10.1182/blood-2002-05-1431.
[442] E.J. Noga, Fish Disease: Diagnosis and Treatment, John Wiley & Sons, 2011.
[443] H. Enocsson, C. Sjöwall, T. Skogh, M.-L. Eloranta, L. Rönnblom, J. Wetterö, Interferon-alpha mediates suppression of C-reactive protein: explanation for muted C-reactive protein response in lupus flares?, Arthritis Rheum. 60 (2009) 3755–3760. doi:10.1002/art.25042.
[444] T. Nakayama, S. Sonoda, T. Urano, T. Yamada, M. Okada, Monitoring both serum amyloid protein A and C-reactive protein as inflammatory markers in infectious diseases., Clin. Chem. 39 (1993) 293–297.
[445] P. Tourlomoussis, P.D. Eckersall, M.M. Waterson, S. Buncic, A comparison of acute phase protein measurements and meat inspection findings in cattle, Foodborne Pathog. Dis. 1 (2004) 281–290. doi:10.1089/fpd.2004.1.281.
[446] J.L. Winkelhake, M.J. Vodicnik, J.L. Taylor, Induction in rainbow trout of an acute phase (C-reactive) protein by chemicals of environmental concern, Comp. Biochem. Physiol. C. 74 (1983) 55–58.
[447] S.Z. Shapiro, S.J. Black, Identification of an acute-phase reactant in murine infections with *Trypanosoma brucei*, Infect. Immun. 60 (1992) 3921–3924.

[448] R.P. Allaker, Host defence peptides—a bridge between the innate and adaptive immune responses, Trans. R. Soc. Trop. Med. Hyg. 102 (2008) 3–4. doi:10.1016/j.trstmh.2007.07.005.
[449] A.L. Coelho, C.M. Hogaboam, S.L. Kunkel, Chemokines provide the sustained inflammatory bridge between innate and acquired immunity, Cytokine Growth Factor Rev. 16 (2005) 553–560. doi:10.1016/j.cytogfr.2005.03.004.
[450] G.S. Getz, Bridging the innate and adaptive immune systems, J. Lipid Res. 46 (2005) 619–622. doi:10.1194/jlr.E500002-JLR200.
[451] J.M. Mansfield, D.M. Paulnock, G.M. Hedberg, Bridging innate and adaptive immunity in African Trypanosomiasis, in: S. Magez, M. Radwanska (Eds.), Trypanos. Trypanos., Springer Vienna, 2014: pp. 89–114. http://link.springer.com/chapter/10.1007/978-3-7091-1556-5_4 (accessed September 6, 2015).
[452] S. Panda, J.L. Ding, Natural antibodies bridge innate and adaptive immunity, J. Immunol. 194 (2015) 13–20. doi:10.4049/jimmunol.1400844.
[453] P.A. Raj, A.R. Dentino, Current status of defensins and their role in innate and adaptive immunity, FEMS Microbiol. Lett. 206 (2002) 9–18. doi:10.1111/j.1574-6968.2002.tb10979.x.
[454] H. Hawlisch, J. Köhl, Complement and Toll-like receptors: key regulators of adaptive immune responses, Mol. Immunol. 43 (2006) 13–21. doi:10.1016/j.molimm.2005.06.028.
[455] M.C. Carroll, The complement system in B cell regulation, Mol. Immunol. 41 (2004) 141–146. doi:10.1016/j.molimm.2004.03.017.
[456] C. Kemper, J.P. Atkinson, T-cell regulation: with complements from innate immunity, Nat. Rev. Immunol. 7 (2007) 9–18. doi:10.1038/nri1994.
[457] D.T. Fearon, R.M. Locksley, The instructive role of innate immunity in the acquired immune response, Science. 272 (1996) 50–54. doi:10.1126/science.272.5258.50.
[458] R. He, L.W. Shepard, J. Chen, Z.K. Pan, R.D. Ye, Serum Amyloid A is an endogenous ligand that differentially unduces IL-12 and IL-23, J. Immunol. 177 (2006) 4072–4079.
[459] J.L. Banyer, N.H. Hamilton, I.A. Ramshaw, A.J. Ramsay, Cytokines in innate and adaptive immunity, Rev. Immunogenet. 2 (2000) 359–373.
[460] R. Clark, T. Kupper, Old meets new: The interaction between innate and adaptive immunity, J. Invest. Dermatol. 125 (2005) 629–637. doi:10.1111/j.0022-202X.2005.23856.x.