Pathophysiology in fish trypanosomiasis: The interaction between *Trypanosoma carassii* and goldfish erythropoietic system during the course of infection

by

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ABSTRACT

Trypanosoma carassii is a hemoflagellate parasite that infects a variety of cyprinid fish. The primary pathophysiology of *T. carassii* infections in fish is the onset of a prolonged anemia, characterized by significant decreases in the number of circulating red blood cells during peak parasitemia. Erythropoiesis is the physiological process which produces new red blood cells and is an essential component in recovery from infection. Previous work in mammalian model systems has suggested that parasite-mediated changes to the immune and erythropoietic systems play a significant role in host anemia. However, the mechanism(s) by which *T. carassii* induces anemia during the course of infection remain to be elucidated.

The gene expression profile of critical erythropoiesis regulators was measured during the course of infection with *T. carassii*, as well as in fish made anemic using phenylhydrazine. Significant upregulation of pro-erythropoietic genes was observed in chemically induced anemia, but not during peak parasitemia. Furthermore, the expression of proinflammatory cytokines was markedly different between chemically and parasitically induced anemia.

A recombinant goldfish erythropoietin (rgEPO) was shown to promote erythroid colony formation *in vitro*. EPO is the main regulator of erythropoiesis and was shown to be downregulated during the early and acute stages of infection. To examine whether the modulation of key erythropoietic factors was responsible for the observed anemia, I administered rgEPO *in vivo* during the early and acute stages of the infection. The administration of rgEPO *in vivo* reduced the severity of anemia but did not restore erythrocyte numbers in infected fish.

Proinflammatory cytokines were upregulated during *T. carassii* infection, specifically tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ). To examine whether these cytokines influence erythropoiesis, progenitor cells were incubated with recombinant goldfish IFNγ and TNFα. Addition of both of these cytokines was shown to suppress the development of erythroid progenitor cells *in vitro*.

My masters research examined the host-parasite interaction between goldfish and *T. carassii*, specifically focusing on the interplay between anemia, erythropoiesis, and inflammation. The results of my thesis research suggest that host proinflammatory responses, combined with direct red blood cell damage induced by *T. carassii,* are involved in the onset and maintenance of anemia during infection.

PREFACE

This thesis is the original work by Mark McAllister and conducted under the supervision of Dr. Miodrag Belosevic, Department of Biological Sciences, University of Alberta. 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).

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The research presented in this thesis represents the analysis of the erythropoietic system of goldfish in response to infection with a protozoan parasite. I was responsible for data collection, analysis and writing of the manuscript. N. Phillips assisted with collection of tissue samples and hemoglobin assays, and also contributed to manuscript edits. M. Belosevic was the supervisory author and involved in formation of concepts and writing of the manuscripts.

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CHAPTER 1

GENERAL INTRODUCTION

1.0 Background

Hematopoiesis is a fundamental physiological process conserved throughout vertebrates that produces a variety of cells that serve a multitude of functions (1). A hierarchical lineage arises from successive waves of hematopoiesis, beginning with hematopoietic stem cells (HSCs) at the onset. HSCs are multipotent cells that are the starting point for all cell types in organisms, and through a series of complex signalling and regulatory processes they eventually differentiate into different cell lineages (2). Erythropoiesis is a branch of hematopoiesis responsible for the production of erythrocytes (red blood cells – RBCs). The key regulator of erythropoiesis is the growth factor called erythropoietin (EPO) (3). Deviations in this process could severely impact the overall fitness of the organism, therefore this process is tightly regulated and responds rapidly to return the host to homeostasis. Erythropoiesis in fish has been well explored, however, regulators that negatively influence this process, specifically parasitic infections, have not been thoroughly investigated.

Parasites are ubiquitous in the aquatic environment and can be found in almost any tissue of the host ranging from intracellular to the exterior surface of the host. Pathogen invasion disturbs homeostasis and can lead to significant alterations to various hematopoietic pathways (4). Impairment of erythropoiesis can lead to the development of anemia, which is a state where there is an imbalance between the production and clearance of erythrocytes. This can impact proper development of an organism, and in severe cases

lead to a state of oxygen deprivation known as hypoxia which can be lethal (5). Additionally, disruptions in hematopoiesis can make the host more vulnerable to secondary infections with viral or bacterial pathogens, which can also lead to high morbidity and mortality (6). In aquaculture setting, disruption in erythropoiesis and thus generation of functional RBCs can cause significant economic losses. In order to mitigate these losses, research has focused on expanding knowledge pertaining to host parasite interactions specifically in the realms of prevention and treatment.

Of specific interest for this thesis research is the aquatic parasite *Trypanosoma carassii* (=*T. danilewskyi*), which is a hemoflagellate that is known to infect a wide variety of economically important fish. The infections with this extracellular protozoan hemoflagellate, can be highly prevalent (100% prevalence in some cases), and can significantly influence host morbidity and mortality (7). Our lab has developed an *in vitro* cultivation system for *T. carassii,* and cultured parasites when injected intraperitoneally into goldfish have been shown to be infectious (8). The ability of *Trypanosoma* spp. to impair erythropoiesis and induce anemia has been extensively studied, and *T. carassii* has been previously shown to reduce the total number of RBCs during infection. Due to the highly conserved nature of the erythropoietic system, the goldfish - *T. carassii* host parasite association is an excellent model to study the mechanisms of pathophysiology in trypanosomiasis.

My thesis research examined the physiological perturbations caused by *T. carassii* infection, specifically focusing on the interplay between the host erythropoiesis and the parasite. By studying these host-parasite interactions during the course of infection, I hoped

to understand how host responses contribute to the control and clearance of parasites, and also shed light on how *T. carassii* modulated these host responses.

1.1 Objective of the Thesis

The specific goal of my thesis research was to examine the erythropoietic system of goldfish during the course of infection with *T. carassii* in the goldfish. The main objective of my thesis research was to analyze the onset and maintenance of parasite-induced anemia. The specific aims of my thesis research were: (a) To analyze changes in hematopoietic regulator gene expression during the course of infection with *T. carassii*; (b) To examine effects of recombinant goldfish erythropoietin (rgEPO) administration both with and without *T. carassii* present; and (c) to examine the effects of exposure to host and parasite derived factors on terminal erythrocytes and erythroid progenitor cells *in vitro.*

1.2 Outline of Thesis

This thesis is comprised of 6 chapters. Chapter 2 is the literature review, which provides a summary of the erythropoietic system in fish specifically focusing on anemia and the relationship between erythropoiesis and trypanosome infections. Chapters 3, 4 and 5 contain findings from my thesis research, including the methods that I used for each group of experiments. Chapter 6 is the general discussion of research findings in the context of my overarching research question.

CHAPTER 2

LITERATURE REVIEW

2.0 Introduction

Hematopoiesis is an extensive and highly regulated process that encompasses all cell lineage pathways including myelopoiesis, lymphopoiesis, and erythropoiesis. Teleost hematopoiesis occurs primarily in the head kidney, which is the functional equivalent to mammalian bone marrow (1). The immune system is intrinsically linked with the hematopoietic system, since immune cells and the products they secrete play an essential role in host immunity. Pathogen challenge can significantly alter proper functioning of both the hematopoietic and immune systems which are critical for maintaining homeostasis. Dysregulation during infection can lead to uncontrolled growth of the pathogen, which can be lethal to the host. This literature review focuses on the interaction between *Trypanosoma carassii* and erythropoiesis.

2.1 Trypanosomes

2.1.1 Mammalian trypanosomes

Trypanosomes are protozoan hemoflagellate parasites belonging to the order Kinetoplastida (Family Trypanosomastidae) (9). Members of this group are characterized by a small disc shaped structure located within the mitochondria known as the kinetoplast. Trypanosomes are generalist parasites, capable of infecting organisms from all vertebrate classes. They are separated into two groups (Salivaria and Stercoraria) based on development within the vector. Salivaria are classified through development in the anterior

portion of the digestive tract and are inoculated into the next host generally through a blood meal. Stercoraria develop in the hindgut of the vector and are transmitted through the posterior end of the host such as excrement. Both groups of trypanosomes are capable of crossing species barriers and are documented to cause medically relevant zoonotic infections in humans.

Trypanosoma brucei contains three subspecies: *T. brucei rhodesiense, T. brucei brucei,* and *T. brucei gambiense*. These species are morphologically indistinguishable, and instead are classified based on their geographical location and host species they infect. *T. b. gambiense* is located in central and West Africa, while *T. b. rhodesiense* is mainly found in East and Southern Africa. Both species are responsible for causing African sleeping sickness in humans, a neuroinflammatory disease that is fatal without treatment (10). *T. b. brucei* is present throughout Sub-Saharan Africa, and infects ruminants resulting in a disease called "nagana" characterized by extreme emaciation and anemia in the vertebrate host (11). *T. brucei* sp. are transmitted by tsetse fly (*Glossina* sp.) and exist exclusively as extracellular organisms in the blood, lymph nodes and spleen of the host (12). These parasites have adapted to this vulnerable environment by utilizing a mechanism known as antigenic variation. This involves continually shifting the expression of the Variable Surface Glycoproteins (VSGs), which are dominant proteins of the outer surface ("coat") of the parasite in order to avoid immune clearance (13). *T. vivax* and *T. congolense* are closely related species that are also responsible for causing nagana. Pathogenesis and life cycle are very similar to *T. brucei* sp., with the major differences being morphology and geographic distribution (14).

T. cruzi is the etiological agent of Chagas disease, or American trypanosomiasis. These parasites are transmitted to the vertebrate host by insect vectors (*Triatoma infestans, T. dimidiata* and *Rhodnius prolixus*), and infect over 150 species of mammals including humans. Unlike the African trypanosomes, *T. cruzi* possesses an intracellular stage that is required for reproduction (15). Spleen, liver and cardiac, smooth and skeletal muscle cells are common targets of the parasite; however, all nucleated cells are capable of being infected. Binding of *T. cruzi* to the cell surface via glycosylphosphatidylinositol (GPI) glycoproteins results in the formation of a parasitophorous vacuole that eventually fuses with the lysosome (16). The acidification of this vacuole induces the production of an acid-activated porin-like molecule that leads to escape of the parasites from the vacuole and differentiation into the intracellular amastigote form (17). This mechanism provides protection from extracellular immune responses, as well as intracellular antimicrobial functions. Acute infection is generally asymptomatic due to small parasite load, however chronic infections can lead to cardiomegaly and eventual heart failure due to prolonged myocarditis caused by parasite invasion into the cardiac muscle tissue (15).

2.1.2 Fish trypanosomes (*Trypanosoma carassii*)

Trypanosoma carassii (=*T. danilewskyi*) is a parasitic hemoflagellate that naturally infects teleost fish including tench, goldfish, and carp (18). T. *carassii* is transmitted from fish to fish by blood-sucking leeches, such as *Hemiclepsis marginata*, which take up parasites in their blood meal (19). Following replication in the crop, parasites migrate to the leech proboscis and are transmitted to the fish host during subsequent feeding (20). *T. carassii* can reach up to 100% prevalence and can cause significant host morbidity and mortality in aquaculture setting (7,21). Pathologies reported during experimental infections

include anorexia and damage to hematopoietic organs such as the kidney and spleen (22,23).

Experimental infection of goldfish (*Carassius auratus* L.) with *T. carassii* results in peak parasitemia at about 3-4 weeks post inoculation. Following this peak, there is a steady decline in parasitemia that results in either clearance of the infection and production of immunity from subsequent parasite challenge, or the development of a chronic infection characterized by low, but persistent, parasitemia (24). Most fish are able to control parasite levels to below a level that is readily detectable ($<5x10^3$ parasites/mL of blood) after 15 weeks. However, in certain cases parasites can be detected up to a year after infection (8). The discrepancy between these time frames, and the mechanism through which these parasites are able to persist in the teleost host remain unclear, however it has been suggested that *T. carassii* is capable of inducing an immunosuppressive state to evade immune clearance (25). Naïve carp that received purified IgM from recovered carp showed altered progression of parasitemia and were able to control the infection (8). Additionally, goldfish that received injections of parasite excretory/secretory (ES) products exhibited decreased parasitemia and administration of recombinant β-tubulin was able to partially protect goldfish from reinfection (26,27). Previous studies in rainbow trout (*Oncorhynchus mykiss*) have shown that it takes approximately 4 weeks to generate high titers of specific antibodies following primary exposure to pathogens (28)

2.2 Anemia

Anemia in fishes is difficult to identify and define, due to the fact they are poikilotherms and blood metrics can fluctuate greatly depending on the external environment. As a result, anemia in fish is defined as a decrease in blood metrics (red blood

cell (RBC) counts, hemoglobin (Hb) concentration and/or packed cell volume (PCV) in comparison to a control group of organisms that are unaffected by a specific treatment (29).

2.2.1 Chemical agents that alter blood metrics and cause anemia

Chemical agents that are capable of negatively impacting the physiology of aquatic organisms have been extensively studied (30–34). Some of these toxic agents are able to directly induce anemia in aquatic organisms, through a variety of mechanisms. Nitrites are pervasive in freshwater aquaculture due to high stocking densities of fish and the excessive use of high protein feed (35). Ammonia is excreted through the gills, and then converted to nitrites by bacteria (36). These nitrites then cross the gill barrier and accumulate in the plasma and subsequently enter tissue and blood cells of fish. Once inside erythrocytes, nitrites can oxidize hemoglobin into methemoglobin and induce oxidative stress leading to hemolytic anemia (37,38).

Metals are also responsible for causing anemia in fish, and in many cases anemic state is one of the earliest indicators of metal toxicity (39). Mercury and mercuric chloride have been shown to drastically reduce RBC numbers, Hb concentration and PCV values in freshwater fish (40,41). Exposure to copper has been shown to increase erythrocyte fragility, making the cells more susceptible to hemolysis (42,43). Long term exposure of freshwater fish to copper, at concentrations ranging from 0.2-0.36 mg/mL, has also been shown to decrease Hb, number of RBCs and PCV (44). Chromium (Cr-VI) forms reactive intermediates that can impact the cellular integrity of erythrocytes and cause hemolysis leading to anemia (45). The effects of chromium on fish hematology have been well established, and was shown to significantly reduce most blood parameters examined in

several different freshwater fish species (46,47). Cadmium has been shown to directly inhibit erythropoiesis in carp (*Cyprinus carpio*) and goldfish (*C. auratus*) by inducing apoptosis of hematopoietic precursor cells (48–50). In addition to its impacts on precursor cells, cadmium has been shown to affect mature erythrocytes. Exposure of carp to 10 mg/L of cadmium resulted in abnormalities in the erythrocyte nucleus and protrusions in the cell membrane (51). Rainbow trout (*Salmo gairdneri*) exposed to varying concentrations of cadmium (4-36 μg/L) showed significant reduction in the total number of circulating mature erythrocytes (52). Freshwater catfish (*Clarias batrachus*) exposed to both lead (0-200 mg/L) and arsenic (1 mg/L) showed decreases in both number of RBCs and PCV in addition to an increase in mean corpuscular volume (MCV) (53,54).

2.2.2 Infectious agents that cause anemia in fish

2.2.2.1 Bacteria

Infection with gram-negative bacteria can lead to severe anemia in teleost fish (55). *V. anguillarum* infection in chinook salmon (*Oncorhyncus tshawytscha*) results in significant reductions in PCV (34% decrease), total RBC (28% decrease), and hemoglobin (32% decrease) (56). The cause of this anemic state was suggested to be either an impairment of the erythropoietic system or accelerated RBC destruction. More recent work with *V. harveyi* suggests that vibriosis-mediated anemia is caused by the production of a potent hemolysin (57). Incubation of *V. harveyi* hemolysin with erythrocytes caused tubular protrusions in the erythrocyte membrane as well as disruptions in the nuclear membrane, ultimately leading to lysis of erythrocytes. Hemolysins produced by other bacterial species has been shown to mediate intravascular hemolysis (58). Goldfish and carp infected with

Aeromonas hydophila exhibit significant decreases in PCV and total number of RBCs, similar to what has been reported for *Vibrio* spp. (59–61). Increased erythrocyte fragility has been reported in rainbow trout (*O. mykiss*) infected with *Pseudomonas putida* leading to global decreases in all measured blood parameters (62). In addition to direct erythrocyte lysis, these bacteria can cause inflammation and liquefactive necrosis of the kidney and spleen which can impair the erythropoietic capacity of the host and cause nonregenerative anemia (55). Bacterially induced anemia is not solely limited to the gram-negative bacteria listed above. Infection of fish with gram-positive bacteria, such as *Streptococcus iniae,* has been shown to cause hemolytic anemia which leads to significant decreases in the number of RBCs and hemoglobin concentration (57).

2.2.2.2 Viruses

Viral diseases can affect hematopoietic tissue and impair production of new erythrocytes. In addition, viruses are capable of infecting nucleated erythrocytes and can directly cause hemolysis. This is a unique aspect of viral infection in teleost fish, as mammalian hosts do not possess nucleated erythrocytes, and therefore viral infections generally result in secondary anemia due to global dyshematopoiesis (64). Viral Erythrocytic Necrosis (VEN) is an iridovirus with a global distribution that infects a wide variety of both economic and non-economic fish including chum salmon (*O. kisutch*), Atlantic cod (*Gadus morhua*), and pacific herring (*Clupea harengus allasi*) (65). The infection of fish with VEN causes significant decreases (~75%) in PCV, followed by a slight increase following clearance of the infection (66). Mature erythrocytes in the vasculature are the main target of the virus in the majority of hosts, however, immature

erythrocytes have been shown to be infected as well (67). Hemolysis is caused by the formation of inclusion bodies in the cytoplasm of the infected erythrocyte, which leads to the eventual degradation of the nuclear and cellular membranes (68). A similar, but distinct, pathophysiology has been reported for Erythrocyte Inclusion Body Syndrome (EIBS) in a variety of salmonid species (58). However, unlike VEN, inclusion bodies of EIBS are mainly observed in immature erythrocytes. Previous studies in Coho salmon (*O. kisutch*) have reported significant reductions in PCV and total number of immature erythrocytes resulting from cellular lysis and immune clearance of infected erythrocytes (69).

Viral Hemorrhagic Septicemia Virus (VHSV) is a viral disease that indirectly causes anemia by damaging hematopoietic tissue. VHSV is rhabdovirus that infects a range of both freshwater and marine hosts including Chinook (*O. tshawytscha*) and Coho (*O. kisutch*) salmon, brown trout (*S. trutta*) and rainbow trout (*O. mykiss*) in North America (70,71). The virus is transmitted via the gill epithelium of the infected fish, and once it enters a naïve host it has a particular tropism for endothelium and certain leukocytes such as macrophages (72). Endothelium tropism leads to the formation of necrotic lesions in tissues throughout the body and can cause extensive damage to the kidney and spleen, which are the primary hematopoietic organs (73). Previous studies have reported significant reductions in erythropoiesis in fish infected with VHSV, including reduced number of immature erythrocytes and impaired development of erythroid progenitor cells (74). Downstream effects of this impairment are manifested as reductions in key blood metrics, including total number of RBCs, PCV, hemoglobin concentration, and mean corpuscular volume (71). Another virus infection that causes similar pathology to VHSV is Infectious Hematopoietic Necrosis Virus (IHNV), which also causes severe necrosis to the

hematopoietic tissues of infected fish, resulting in secondary anemia due to insufficient restoration of the RBC pool (75–77).

2.2.2.3 Protozoa

A variety of protozoan parasites are capable of existing both intra- and extracellularly within teleost fish. *Cryptobia salmositica (=Trypanoplasma salmositica*) is a extracellular hemoflagellate which has been reported to cause anemia in all species of salmon in North America (18). Severity of anemia was directly correlated with intensity of infection, whereas pathogenicity was determined primarily by measuring mortality rates that ranged from 100% in chinook salmon, to 0% in Coho salmon (78). The agents causing anemia during *C. salmositica* infection are a parasite-derived cysteine protease and a metalloprotease (79). These proteins are secreted by living parasites into the vasculature of the host, where they display proteolytic and hemolytic effects (80). The metalloprotease was shown to specifically target proteins in the cellular membrane of erythrocytes of rainbow trout, leading to lysis of the RBCs (81). Similar pathology has been reported to be caused by *Trypanoplasma borreli,* a related parasite that also causes progressive anemia and extensive damage to hematopoietic tissues of infected fish (82). Carp expressing an anti-metalloprotease protein experienced significantly reduced morbidity when infected with *T. borreli*, suggesting that the mechanism behind this anemic state may be mediated by a metalloprotease similar to that identified in *C. salmositica* (83). Additionally, studies using Atlantic salmon (*S. salar*) experimentally infected with *Spironucleus barkhanus* have reported significant reductions in PCV along with lesions in hematopoietic tissues (84).

While overall pathophysiology resembles that of *T. borreli* and *C. salmositica,* the mechanism behind this observed anemia caused by *S. barkhanus* remains unclear.

Intraerythrocytic parasites are common pathogens of teleost fish, however relatively few studies have reported anemia as a secondary pathology of infection (85). Severe anemia has been observed in chinook salmon (*O. tshawytscha*) infected with *Microsporidium rhabdophilia*, a intracellular parasite that causes cell lysis through the formation of eosinophilic inclusion bodies (86). PCV was inversely related to the percentage of hemoblastic cells presenting with inclusions, with the most severe infections resulting in 10-fold reductions in PCV (86). Similar pathophysiology has also been described for other hatchery-reared fish including; Coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*) (87,88).

2.2.3 Anemia caused by trypanosomes

Anemia is one of the most prominent pathophysiological manifestations caused by *Trypanosoma* spp. infections, and has a complex etiology (89). Anemia has been reported during infection with a broad range of fish trypanosomes, including *T. carassii, T. murmanensis, T. batrachi, T. aligaricus,* and *T. attii* (90–92). Collectively, trypanosome infections in fish are generally regarded as innocuous, and do not result in significant mortality (8). Infections are characterized by global decreases in hematological indices including PCV, hemoglobin, and total erythrocyte counts (93). The mechanism behind the induction of anemia remains to be fully elucidated for any piscine trypanosome species. However, previous studies examining *T. carassii* infection in goldfish has suggested that this anemic state may be due to the release of a parasitic excretory/secretory hemolysin

molecule, similar to what has been observed for other non-trypanosomatid parasites such as *C. salmositica* and *T. borreli* (23,78,82).

Trypanosome-mediated anemia in mammalian models has been more extensively explored, and several mechanisms have been previously described. The production of a hemolysin molecule, similar to what has been described in teleost models, has been documented for *T. cruzi* which produces an acid-activated hemolysin that functions at low pH (94). It was predicted that this molecule was utilized as a means of escape from acidic vacuoles during the intracellular life cycle of the parasite. A hemolysin capable of inducing intravascular hemolysis has been reported to be produced by *T. cogolense* and *T. brucei* while the parasites were actively dividing in circulation (95,96). Hemolytic activity was found to be mediated primarily by linoleic acid, which possess detergent-like qualities and was strongly hemolytic to erythrocytes (97,98). This hemolytic activity was further supplemented by the release of phospholipases following parasite autolysis (99). These phospholipases generated several other free fatty acids, which acted in an identical fashion to linoleic acid. Additionally, *T. congolense* has been previously reported to produce a hemolysin known as thiolprotease which was capable of cleaving various substrates including those found in erythrocyte membranes that contributed to intravascular hemolysis (100).

T. congolense and *T. evansi* have been shown to produce neuraminidase, which functions by cleaving sialic acid residues on the surface of erythrocytes, which modified the cells and caused them to be targeted by the immune system (phagocytosis) (101). These findings were corroborated by recent work using *T. vivax*, that showed that erythrocyte desialyation alone lead to the induction of significant anemia via erythrophagocytosis

(102). It has also been suggested that in addition to specifically modifying these cells for destruction, sialidases may interact with erythropoietin by desialyating this RBC growth factor, which may reduce overall biological activity and plasma half-life (103).

Parasite growth leads to the production of metabolic by-products which can damage erythrocyte membranes and induce the production of reactive oxygen species (ROS) (104). The generation of ROS by erythrocytes leads to lipid peroxidation, which is a process in which free radicals and super oxides are formed through reduction/oxidation reactions with polyunsaturated fatty acids and proteins in the cell membrane (105). Free radicals and super oxides are also produced by the immune respiratory burst response in activated macrophages and neutrophils during infection (106). Overproduction of these oxidative products leads to decreased erythrocytic membrane integrity and eventually oxidative hemolysis. Previous studies in mice infected with *T. brucei* have reported significantly increased production of oxidative products such as lipid hydroperoxide and malonaldehyde (107). This increase was directly correlated with decreases in PCV. Similar results were reported in sheep infected with *T. brucei*, where erythrocytes became damaged in response to peroxidation (108).

Increased expression of proinflammatory genes during the course of infection has been demonstrated in various model systems including *T. cruzi* and *Leishmania* spp. (109,110). Proinflammatory cytokines act primarily as indirect mediators of anemia in trypanosomiasis by inhibiting hematopoiesis. Of specific importance are interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) which are central regulators of inflammation and have been previously implicated in controlling the course of trypanosomiasis (111,112). TNFα has been shown to directly contribute to anemia by

accelerating erythrocyte clearance through increased erythrophagocytosis, and indirectly by inhibiting proliferation and differentiation of erythroid progenitor cells (113,114). IFNγ on the other hand, directly induces anemia by altering iron metabolism (115). Iron plays an essential role in hematopoiesis, specifically erythropoiesis (116). During homeostasis serum iron levels are tightly regulated by transferrin, however, following infection the antimicrobial peptide hepcidin functions to limit free iron for pathogens (117). IFNγ increases iron uptake in activated macrophages, and promotes storage of the ferrous iron into ferritin (118). Sustained sequestration of iron leading to low serum levels can negatively impact erythropoiesis by depriving progenitor cells of iron necessary for proper erythrocyte development, which may lead to progressive anemia (116) Additionally, IFNγ has been shown to inhibit erythroid cell development *in vitro* by promoting apoptosis of hematopoietic progenitor cells (119).

2.3 Erythropoiesis

Erythropoiesis is a branch of hematopoiesis and serves as the mechanism by which red blood cells (RBCs) are generated. The main erythropoietic organ in teleost fish is the kidney, and to a lesser extent, the spleen (120).The steps in the process are complex, but one of the key features of erythropoiesis is the binding of RBC growth factor, erythropoietin (EPO), to its cognate receptor EPOR. Human EPO was first cloned in 1985, and since then homologous genes have been identified in a wide range of mammalian species, as well as several fish species. The first teleost EPO molecule was cloned from pufferfish (*Fugu rubripes*), and was then subsequently identified and cloned in zebrafish and goldfish (121–123).

2.3.1 Teleost erythropoiesis

The process of erythropoiesis is highly conserved throughout vertebrate evolution, with many key regulators sharing identical functions during cell development. Hematopoietic stem cells (HSCs) are the primary progenitor cells that give rise to all cell types (124). Following stimulation by different cytokines, multipotent HSCs then differentiate into either the erythroid/myeloid (giving rise to erythrocytes, thrombocytes, granulocytes, and monocytes) or lymphoid lineage (giving rise to B-cells, T-cells, natural killer (NK) cells, and dendritic cells) (125). In the kidney of fish, common myeloid progenitors differentiate into megakaryocyte/erythroid progenitors following stimulation by a specific combination of factors, generally consisting of stem cell factor (SCF) and interleukins/growth factors (126). Specific differentiation into erythroid progenitor cells occurs first via burst-forming unit-erythroid (BFU-E) and then colony-forming unit-erythroid (CFU-E) cells (127). Following stimulation with EPO and Kit ligand (Kitl), CFU-E cells differentiate into erythroblast cells, which differentiate into terminal erythrocytes following stimulation with EPO (128).

2.3.2 Teleost erythropoietin

In mammals, EPO is constitutively produced in the kidney and production is increased in response to anemia and hypoxia (3). Expression of EPO in teleost models has also been shown to be upregulated in response to anemia, similar to what has been observed in mammals (129,130). Unlike mammals however, multiple organs contribute to EPO production including the heart, liver, kidney and spleen (122). Following production EPO

binds to, and causes homodimerization of, EPOR which is primarily located on the surface of erythroid progenitor cells. Signal transduction then occurs through the JAK2/STAT5 pathway, which upregulates anti-apoptotic genes and promotes cell survival (131). Knockdown of JAK2 in zebrafish results in significant reductions in the number of terminal erythroid cells. Conversely, injection of a constitutively active form of JAK2 leads to increase in erythropoiesis; suggesting that signalling through the EPOR is essential for proper erythropoiesis (132).

2.4 Mechanisms of immunity in trypanosomiasis

2.4.1 Role of antibodies in *T. carassii* infections of fish

Infection with *T. carassii* can be classified into two main stages: acute and chronic. The acute stage of infection includes the timeframe where parasites are rapidly replicating, and reach peak parasitemia around 21-28 days post infection (24). During this time, hosts that are incapable of successfully controlling the parasites will succumb to the infection with mortality depending on multiple factors including nutrition, stress, and infection with secondary pathogens (133,134). If the host is able to effectively control infection with *T. carassii*, then they will enter the chronic stage of infection; generally occurring around 35-49 days post infection characterized by very low levels of parasitemia (135). Hosts that recover from infection generate a non-sterile immunity which protects them from reinfection (18). Previous studies have shown this immunity to be antibody mediated, as passive transfer studies from immune to naïve hosts using immune serum and purified IgM antibodies were capable of conferring protection against reinfection with *T. carassii* (26).

2.4.2 Role of other soluble factors in trypanosome infections

Soluble immune factors encompass a wide range of molecules, including; cytokines, peptides, acute phase proteins, as well as receptors (136). These factors are involved in both the innate and adaptive arms of the immune system, and play a key role in influencing the outcome of an immune response (137).

Cytokines are key immune signalling molecules and are produced in response to pathogen exposure. Binding of cytokines to receptors on the surface of different immune cells can cause differentiate into different subsets with divergent effects (138). Th1 and Th17 cell types regulate cell-mediated immunity and are generally involved in defense against intracellular pathogens. Th2 cells mediate humoral immunity and is generally responsible for controlling extracellular pathogens. Proinflammatory cytokines have been linked to protection against bacterial, fungal, viral and parasitic infections (139). Goldfish infected with *T. carassii* upregulate the expression of proinflammatory cytokines such as TNFα1, TNFα2 IFNγ, IL-1β-1 and IL-1β-2 (111). Both IFNγ and TNFα have been shown to mediate resistance to *Trypanosoma* spp (110,140–142). Previous studies have demonstrated that trypano-tolerant cattle resistant to *T. congolense* infection regulate infection better than trypano-susceptible cattle (Boran) due to higher IFNγ mRNA levels (143). Additionally, TNFα knockout mice have been shown to present with increased morbidity/mortality in response to infection with *T. brucei* (144). Further studies revealed that TNFα was directly involved in inducing trypanocidal responses in hosts infected with *T. cruzi* (145).

Parasite derived factors have been reported to alter the Th1/Th2 balance during infection by influencing proinflammatory responses (146). In *T. carassii* infections, the

parasite was shown to produce glycoprotein 63, which downregulated the production of reactive oxygen and nitrogen intermediates following TNFα2 stimulation (147). Additionally, heat shock protein (hsp70) produced by *T. carassii* was shown to induce a variety of proinflammatory cytokines including IFNγ, TNFα, IL-1β, and IL-12 (148).

2.4.3 Immune evasion mechanisms of trypanosomes

Both intracellular and extracellular species of *Trpanosoma* spp. have developed immune evasion mechanisms that enable them to establish and thrive in their vertebrate hosts. For example, *T. cruzi* utilizes trans-sialidase to cleave host sialic acid residues which are then bound by mucin glycoproteins expressed on the surface of the parasite, effectively shielding the parasite from host immune recognition (149). VSGs are employed by the extracellular *T. brucei* as a method to avoid destruction by the immune system. The parasite possesses a wide variety of genes that encode for antigenically distinct surface proteins, and through a process of allelic exclusion will successively alter the expression of these surface proteins (150). Once an antibody response is generated to a specific VSG, a proportion of the population will begin expressing a new form and quickly re-establish within the vasculature (25).

No intracellular stages have been reported in *T. carassii*, suggesting that this parasite is exclusively extracellular. However, analysis of the parasite surface molecules has revealed that it is most similar to *T.cruzi*, despite the contrasting lifecycle environments. Treatment of *T. carassii* with trypsin to cleave this surface coat resulted in increased susceptibility to antibody and complement-mediated lysis (151). Further investigation revealed the presence of a *T. carassii* calreticulin molecule, that has

previously been shown to bind complement C1q component, and thus effectively inhibited classical-complement mediated trypanolysis (152). It has also been hypothesized that this calreticulin molecule impaired the ability of antibodies to bind and induce an immune response against the parasites (25).

Modulation of the host Th1/Th2 response has been reported to be a potential mechanism of immune evasion in *T. carassii*. Gene expression analyses in goldfish demonstrated that *T. carassii* induced a mixed Th1/Th2 response – upregulating the expression of both pro- and anti-inflammatory genes that are involved in immunosuppression (153). Parasite derived molecules, such as hsp70, were shown to increase the production of proinflammatory cytokines and bias the immune response towards a Th1 type; thereby allowing them to avoid the more detrimental effects of a Th2 (antibody mediated) response during the key establishment phase of the infection (148). In contrast gp63 has been shown to downregulate proinflammatory responses and shift the immune balance back towards a Th2-type response, suggesting that *T. carassii* is capable of altering the host immune response at various stages of the infection in order to remain in the host for prolonged periods.

2.5 Summary

Trypanosoma spp. are generalist parasites that are able to infect a wide variety of species including both endotherms and ectotherms. In these hosts, they exist either intracellularly or extracellularly; and have developed several mechanisms which allow them to adapt and survive in this hostile microenvironment. Modulation of host physiology is critical to ensure parasite survival, and includes several interconnected systems including

the immune system, endocrine system, and hematopoietic system (25). Impairment of the erythropoietic system, which is responsible for producing RBCs, is a notable pathology associated with trypanosome infections and is a major factor in causing host mortality (154). The etiology behind this anemia is multifactorial and arises from a complex interaction between the host and parasite. Trypanosomes have been implicated in the induction of this anemia via the production of factors that cause intravascular lysis and directly suppress erythropoiesis; whereas host-derived factors such as over-induction of proinflammatory responses has been shown to induce and sustain this anemic state and exacerbate host pathophysiology (155).

CHAPTER 3

ANALYSIS OF ERYTHROPOIESIS REGULATOR GENE EXPRESSION IN GOLDFISH (*Carassius auratus)* INFECTED WITH *Trypanosoma carassii*[1]

3.0 Introduction

As previously mentioned, EPO expression is induced in response to situations which require an increase in the number of circulating RBCs, namely anemia and hypoxia. The binding of EPO to its cognate receptor (EPOR) triggers downstream signalling resulting in proliferation and differentiation of terminal erythroid cells, and prevents apoptosis of the developing cell (3). While EPO has been identified as a key regulator in controlling erythropoiesis, other genes involved in terminal cellular development in response to infection have yet to be explored.

The expression of genes involved in hematopoiesis have been highly conserved between mammals and teleost fish (1). Members of the GATA family are a class of transcription factors that function at several stages of hematopoiesis and serve as important upstream regulators of cell differentiation. There are 6 total GATA genes, with GATA-1, GATA-2 and GATA-3 specifically involved in teleost hematopoiesis (156).

GATA-2 is active earliest in the hematopoietic pathway, and has been previously reported to be involved in preventing differentiation of HSCs in order to maintain a pool of

[1] A portion of this chapter has been published: McAllister M, Phillips N, Belosevic M. "*Trypanosoma carassii* infection in goldfish (*Carassius auratus* L.): changes in the expression of erythropoiesis and anemia regulatory genes" *Parasitology Research* vol. 118, issue 4, 1147-1158.

undifferentiated cells (157). GATA-3 expression is heavily involved in lymphopoiesis and is restricted to NK cells and T-cells (158,159). GATA-3 is further affiliated with the immune system by inducing the expression of genes involved in Th2 subtype differentiation and cytokine production (160,161).

GATA-1 on the other hand is tightly linked with differentiation of hematopoietic progenitors into erythroid cells. GATA-1 is active in myeloid progenitor cells, and will initiate the transcription of genes that are essential in erythroid cell development (162). This role is directly antagonized by PU.1, which is a transcriptional activator of a variety of myeloid genes (163). GATA1 is essential for proper erythroid development, as mouse models with a GATA1 knockout demonstrate arrested erythropoiesis, and embryos that are GATA1 deficient die as a result of severe anemia (164). Loss of GATA1 in zebrafish has also been shown not only to severely represses erythropoiesis, but also pushes cells towards development in the myeloid lineage (165). Studies examining the relationship between GATA-1 and GATA-2 have reported conflicting findings. Previous reports have shown GATA-2 to be antagonistic to GATA-1; with induced expression of GATA-2 in progenitor cells causing inhibited erythroid differentiation, and induction of myeloid differentiation (166,167). Others have shown that expression of GATA-2 is able to regulate expression of erythroid development genes in progenitor cells in the absence of GATA-1, and can lead to recovery from *gata1*$^{-/-}$ induced anemia (168).

Lmo2 is another transcriptional regulator that acts as a marker of erythroid development, and is essential for proper erythropoiesis (169). Lmo2 belongs to a class of transcription factors that assemble into a multimeric complex following GATA-1 stimulation (170). Injection of exogenous Lmo2 mRNA, along with its co-regulator SCL,

has been shown to broadly induce erythropoiesis and expand the area in which erythroid progenitors are found within the kidney (171).

Hypoxia inducible factor (HIFα) is a transcriptional regulator that is activated in both mammals and fish in a state of oxygen deprivation, and leads to increased erythropoiesis (172). This occurs by directly binding to the promoter region of EPO to increase expression of the glycoprotein, as well as activating the transcription of other genes involved in externally regulating the erythropoietic pathway (5,173). Finally, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) are proinflammatory cytokines that are upregulated in response to *T. carassii* infection, and have been shown to influence erythropoiesis, and were therefore included in the panel of examined genes (25,113).

In this study, I examined the expression of genes that encode regulators of erythropoiesis both directly and indirectly to assess the impact that infection with *T. carassii* has on this system.

3.1 Methods

3.1.1 Infection of goldfish with *T. carassii*

Parasite cultures of *Trypanosoma carassii* were maintained as glycerol stocks in our lab at -80°C until needed for assays. Thawed stocks were cultured *in vitro* by serial passage through TDL-15 medium supplemented with 10% heat-inactivated goldfish serum, as previously described (27). Parasite cultures were incubated at 20°C and passed every 6-7 days.

Fish were anesthetized with TMS and then injected intraperitoneally using 25GA needle and tuberculin syringe with high dose (HD) of 1 x 10^7 parasites in 100µL of TDL-15 medium. Control fish were injected with 100µL of TDL-15 medium alone.

3.1.2 Treatment with Phenylhydrazine

Goldfish were injected with 12.5µg/g of phenylhydrazine (PHZ), as has been previously described (174). In brief, 100µL of PHZ dissolved in 0.9% sterile saline solution was injected intraperitoneally. Blood and tissue samples were collected at 48 hrs post-injection to determine PCV and RBC number and assess the mRNA levels using quantitative PCR. In preliminary experiments, most significant anemia was observed at 48 hrs post administration of PHZ. Exposed fish recovered within one week, similar to previous reports (174).

3.1.3 Assessment of the impact of *T. carassii* infection on expression of erythropoiesis regulators in goldfish

Whole kidney, liver and spleen tissues were collected from control and infected fish at 4, 7, 14, 28, 42 and 56 days post-infection (dpi); however, gene expression analysis was done using tissues collected 7, 28 and 56 dpi as representative timepoints for the early, acute and resolution stages of infection. Control and treatment groups consisted of 24 fish in replicate tanks, for an n=4 at each timepoint. Each group of 24 fish was further divided into two tanks of 12 fish to mimic population density from cohort infections. Whole kidney, liver and spleen tissues were also collected from PHZ treated fish (n=4) at 48 hours post-injection. Samples were flash frozen in liquid nitrogen, and then stored at -80˚C. RNA

was extracted using a phenol-chloroform extraction method. Two µg of RNA was then reverse transcribed into cDNA using a Superscript III cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. 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 0.970, and efficiency was between 90-110%. qPCR cycling parameters were: 95˚C, 2 min; 95˚C, 15 s; 60˚C, 1 min; 95˚C,15 s; 60˚C,1 min; 95˚C, 30 s; 60˚C,15 s (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), since it is stably expressed in all goldfish tissues, and has been previously used as an endogenous control gene by our laboratory (111,175) and others (176). All samples were run in triplicate and presented as the mean ± SEM of 4 fish for medium control and high dose groups.

3.1.4 Statistical Analysis

qPCR results were analyzed by unpaired multiple *t* test, with a probability of $P<0.05$ considered significant.

3.2 Results

3.2.1 *T. carassii* alters expression of erythropoiesis-associated genes during infection

In the kidney, which contains the main hematopoietic organ the head kidney, the expression of erythropoiesis regulator genes tended to have either reduced or unchanged expression compared to control fish (Figure 3.1A-B). EPO and its receptor EPOR, along

with GATA1 and HIFα, were significantly downregulated at 7 days post infection (dpi) however only EPO and EPOR remained downregulated at 28 dpi. This was followed by an upregulation in the expression of EPO and HIFα at 56 dpi (Figure 3.1C). The mRNA levels of the proinflammatory genes showed contrasting expression profiles. IFNγ remained upregulated during the course of the infection, whereas TNFα showed a significant spike at 28-dpi with a 59-fold increase in mRNA levels (Figure 3.1B).

In the liver, mRNA levels remained relatively static for erythropoiesis-associated genes at 7 and 28 dpi (Figure 3.2A-B). At 56 dpi there was a significant increase in the expression of all erythropoiesis regulator genes in comparison to non-infected control fish, the largest being EPO with an 18-fold increase (Figure 3.2C). mRNA levels of proinflammatory genes followed a similar pattern when compared to that in the kidney, where IFNγ expression remained high throughout the infection and TNFα peaking at 28 dpi before returning to near baseline levels at 56 dpi (Figure 3.2B-C).

In the spleen, mRNA levels were significantly decreased for EPO and GATA1 at 7 dpi, with EPOR and Lmo2 also showing moderate decreases (Figure 3A). EPO mRNA levels remained decreased at 28 dpi, whereas the expression of Lmo2 increased (Figure 3B). Large increases in erythropoiesis regulator mRNA levels, with the exception of EPOR, occurred at 56 dpi. The greatest increases observed were EPO (18-fold), as well as GATA1 (20-fold) (Figure 3C). Expression of IFNγ and TNFα mirrored the trends observed in the kidney and liver, with IFNγ increased throughout and TNFα peaking at 28 dpi with a 34-fold increase in mRNA levels (Figure 3B).

3.2.2 PHZ treatment causes increased expression of erythropoiesis-associated genes

mRNA levels in the kidney, liver and spleen of goldfish were examined following administration of PHZ, a pharmacological inducer of anemia (179,180). The kidney showed increased levels of EPO and its receptor EPOR, along with the transcription factor GATA1 (Figure 3.4A). Similar increases were observed in the spleen, with the addition of HIFα (Figure 3.4C). The liver displayed the greatest changes in EPO expression with a 39-fold increase in mRNA levels at 48hrs post PHZ injection (Figure 3.4B). Unlike in *T. carassii*-infected fish, there were no significant changes in IFNγ mRNA levels after PHZ treatment. However, there was a significant increase in TNFα levels in kidney, liver and spleen following PHZ injection with the most significant increase occurring in the spleen (Figure 3.4C).

3.3 Discussion

The mRNA levels of key erythropoiesis regulator genes at critical points during infection are similar to what has been observed in mammalian model systems. Mice infected with *T. congolense* show reduced expression of both EPO and its receptor EPOR in the liver and kidney during the initial stages of infection while parasite numbers increase drastically (181). Subsequent studies using the same model system have demonstrated that more severe infections are linked to lower expression levels of hematopoietic transcription factors GATA1 and Lmo2 (182). Sudden decreases in circulating erythrocyte numbers generally leads to hypoxia, which induces the production of EPO in the head kidney and subsequently stimulates erythropoiesis in order to replenish peripheral cell numbers (154,183). Progenitor cells require stimulation with EPO in order to activate DNA-binding activity of GATA-1, which then leads to erythroid maturation (184). This decrease was no longer apparent during the resolution phase, which was characterized by a simultaneous

increase in the expression of these genes across all tissues along with an increase in circulating blood cells. This suggests that there is some restraint on erythropoiesis during the acute stages of infection which impairs normal erythrocyte development. This gene expression analysis, in conjunction with previous findings, suggests that this could be due in part to a decrease in EPO. Impaired production of EPO could cause reduced downstream signalling resulting in fewer terminal erythrocytes being produced to replenish basal levels. This expression profile was directly contrasted by the results obtained in chemically induced anemia.

PHZ is commonly used to chemically induce anemia in animal models by increasing ROS, causing oxidative degradation and clearing of RBCs (174). Studies in poikilotherms have shown that erythropoiesis is immediately increased in response to chemically induced anemia, and recovery generally occurs within a week under normal environmental temperatures (185). Increased mRNA levels of EPO have been observed in Atlantic salmon (*Salmo salar* L.) as well as the South African clawed frog (*Xenopus laevis*) in response to PHZ injections (129,186). The increase in the EPO mRNA levels is related to subsequent recovery of PCV and total RBC numbers, suggesting that EPO regulation may be the driving force behind recovery from anemia (187). Gene expression analysis at peak anemia revealed that the levels of EPO mRNA increased in all tissues, most significantly in the liver and spleen, while the expression of IFNγ remained unchanged. Interestingly, TNFα levels increased in response to PHZ injections which contradicts previous findings from a mammalian system (154). Our results indicate that goldfish erythropoietic pathway is immediately and significantly upregulated in response to anemia exemplified by restoration of the RBC numbers, in contrast to what was observed during

infection with *T. carassii*. One of the key differences between these two forms of anemia appears to be the expression profiles of proinflammatory cytokines which are notably different between *T. carassii* infection and PHZ injection.

IFNγ is important in controlling early infections with intra and extracellular protozoans, and has been previously shown to increase during trypanosomiasis (111). The main function of IFNγ during infection in goldfish is to activate macrophages in order to stimulate the production of nitric oxide (NO) (25). However, IFNγ has also been shown to inhibit erythroid cell development *in vitro* by promoting apoptosis of hematopoietic progenitor cells (188). Trypanotolerant N'Dama cattle are able to resist infection due an increased expression of IFNγ, which downregulates the erythropoietic pathway (189). Additionally, TNFα has also been implicated in the etiology of anemia during trypanosomiasis. TNFα plays an essential role in defense against trypanosomes, with deficient mice demonstrating more significant morbidity/mortality in response to infection (190). Additionally, serum TNFα levels have been previously demonstrated to be related to PCV changes in rats infected with *T. evansi* (191). This is thought to be a result of inhibited proliferation and differentiation of erythroid progenitor cells as well as increased erythrophagocytosis; both of which are mediated in part by TNFα (178). TNFα role in erythrophagocytosis may explain the increased expression of TNFα observed following PHZ injection, where reduced membrane integrity may necessitate increased RBC clearance. Expression analysis of *T. carassii* infected goldfish has revealed that *T. carassii* preferentially stimulates a Th1 type immune response, characterized by significant increases in IFNγ, TNFα, and other proinflammatory cytokines during the acute phase of infection (111). Because *T. carassii* is an extracellular parasite, the promotion of a Th1

rather than Th2 response may be a potential immune evasion mechanism. Thus, in addition to its potential role in immune evasion, this observed increase in IFNγ may be partially responsible for inhibiting normal erythropoiesis, leading to the prolonged anemic state observed during *T. carassii* infection.

Table 3.1 List of primer sequences and their function

PRIMER	FUNCTION	SEQUENCE (5' – 3')
EPO Sense EPO Antisense	Erythropoiesis Stimulating Glycoprotein	GGG ATG CAG AGG CTG CTA TG GCT TCC CAG ACA TCA AAA TCA AC
EPOR Sense EPOR Antisense	EPO Cell Surface Receptor	GAC CGA GCC TGT GTT TGG A CAG AGA CAG CAG GCA GAG GAT
GATA1 Sense GATA1 Antisense	Erythroid Transcription Factor	GTC TGT TCT GGC CGT TCA TTT T ACC AGA GGC ACG TGA ATG C
Lmo2 Sense Lmo2 Antisense	Erythroid Transcription Factor	TGC GTG TCC GTG ACA AAG TC AAA AAT GTC CTG CTC ACA CAC AA
HIFα Sense HIFα Antisense	Oxygen Stress Response Transcription Factor	AGA CCT TTC TTA GCC GCC AC CGG GAC ACT TGA GGC TTT CT
IFNγ Sense IFNγ Antisense	Proinflammatory Cytokine	GAA ACC CTA TGG GCG ATC AA GTA GAC ACG CTT CAG CTC AAA CA
TNFα Sense TNFα Antisense	Proinflammatory Cytokine	TCA TTC CTT ACG ACG GCA TTT CAG TCA CGT CAG CCT TGC AG
EF-1 Sense EF-1 Antisense	Endogenous Control	CCG TTG AGA TGC ACC ATG AGT TTG ACA GAC ACG TTC TTC ACG TT

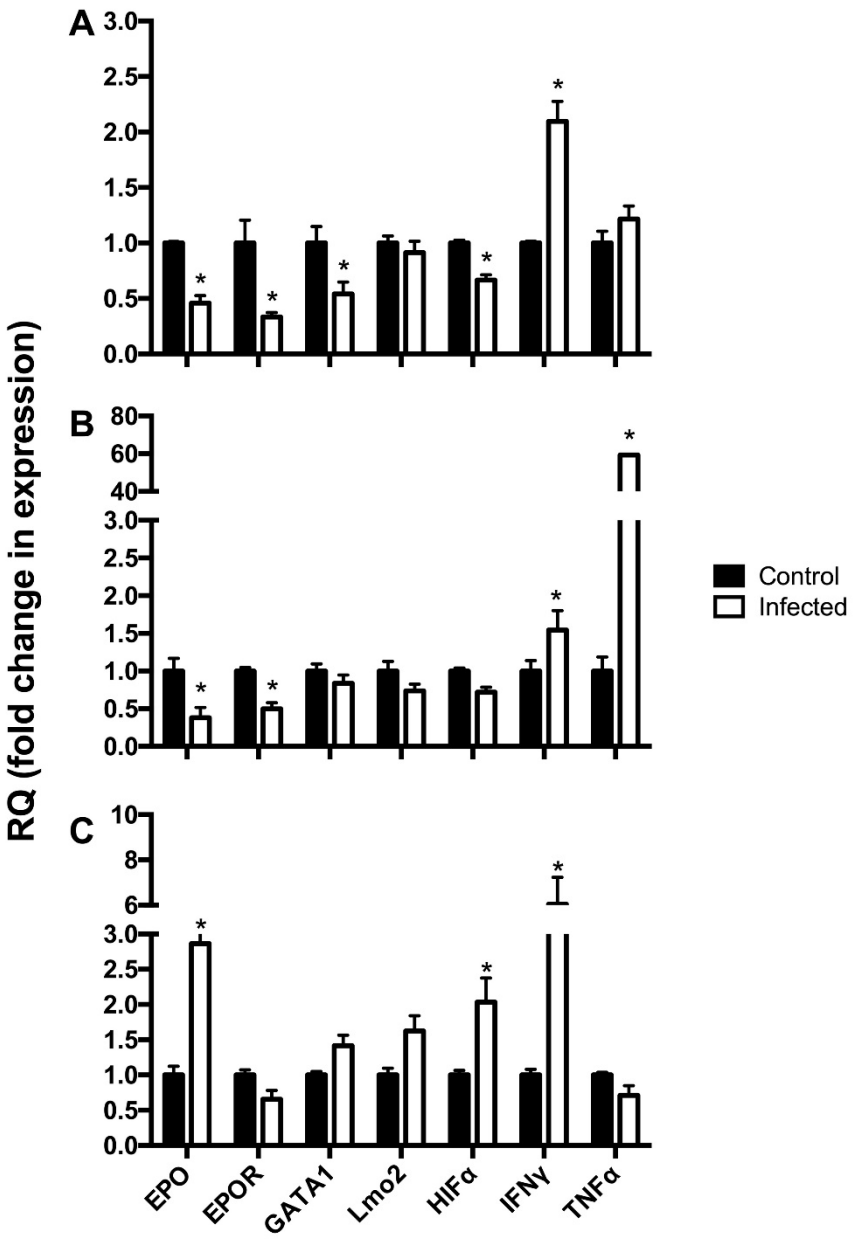

Figure 3.1 Kidney mRNA levels of genes encoding erythropoiesis regulators in T. carassii infected and non-infected goldfish.
(A) 7 dpi (B) 28 dpi (C) 56 dpi. Expression was relative to endogenous control gene, EF-1α and normalized against those observed in non-infected control fish for each gene. Data are represented as mean ± SEM of 4 fish ($P < 0.05$). Asterisks (*) denote statistical significance from non-infected control fish. (EPO – Erythropoietin; EPOR – Erythropoietin receptor; HIFα – Hypoxia Inducible Factor alpha; IFNγ - Interferon gamma; TNFα – Tumor Necrosis Factor Alpha).

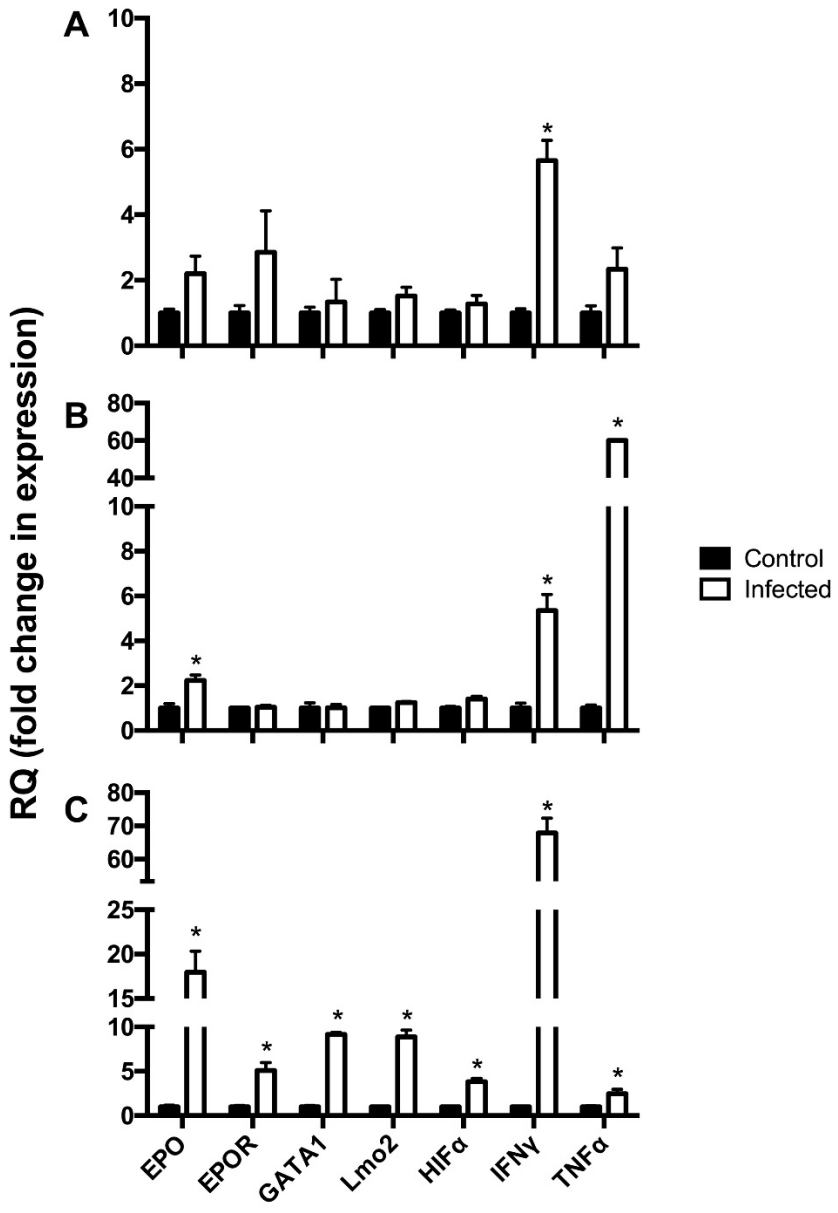

Figure 3.2 Liver mRNA levels of genes encoding erythropoiesis regulators in T. carassii infected and non-infected goldfish. (A) 7 dpi (B) 28 dpi (C) 56 dpi. Expression was relative to endogenous control gene, EF-1α and normalized against those observed in non-infected control fish for each gene. Data are represented as mean ± SEM of 4 fish ($P < 0.05$). Asterisks (*) denote statistical significance from non-infected control fish. (EPO – Erythropoietin; EPOR – Erythropoietin receptor; HIFα – Hypoxia Inducible Factor alpha; IFNγ - Interferon gamma; TNFα – Tumor Necrosis Factor Alpha)

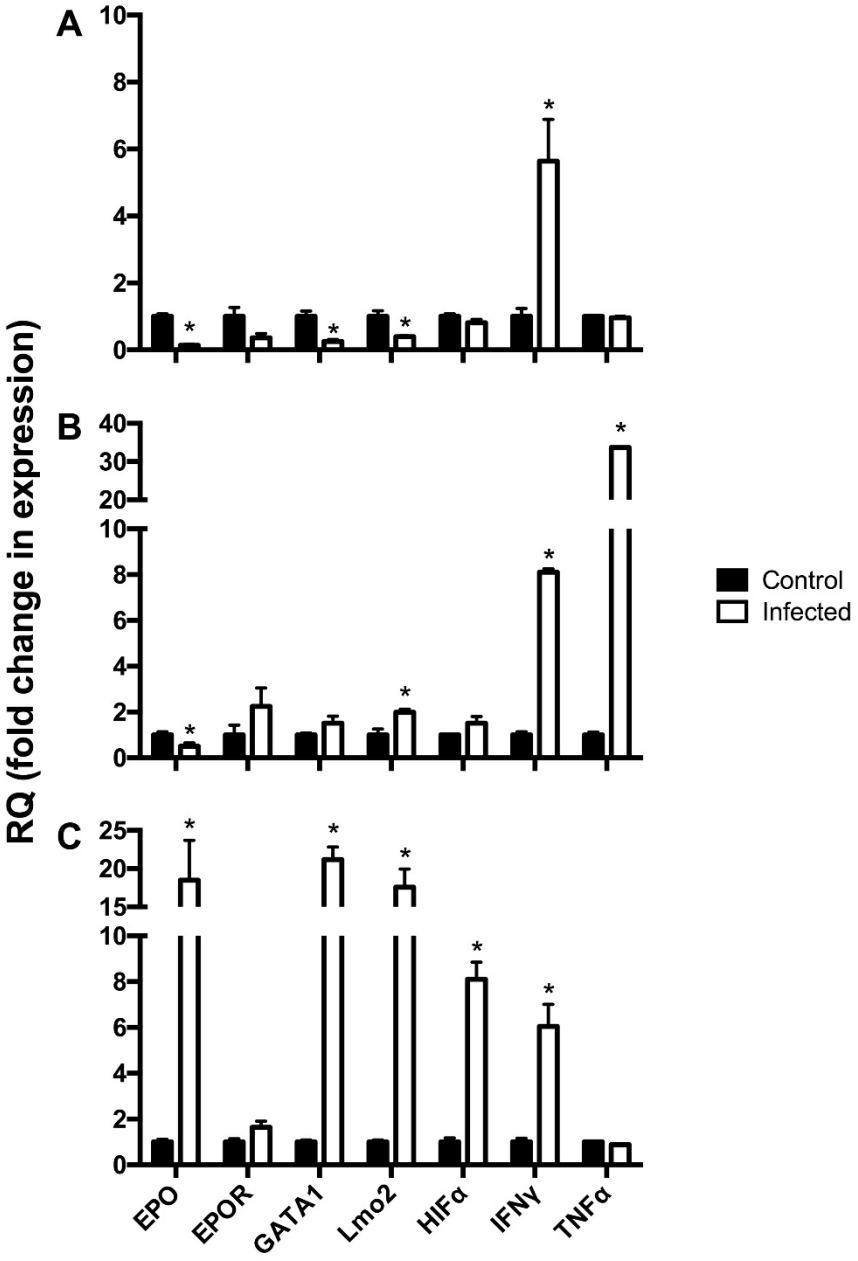

Figure 3.3 Spleen mRNA levels of genes encoding erythropoiesis regulators in *T. carassii* infected and non-infected goldfish.
(A) 7 dpi (B) 28 dpi (C) 56 dpi. Expression was relative to endogenous control gene, EF-1α and normalized against those observed in non-infected control fish for each gene. Data are represented as mean ± SEM of 4 fish ($P < 0.05$). Asterisks (*) denote statistical significance from non-infected control fish. (EPO – Erythropoietin; EPOR – Erythropoietin receptor; HIFα– Hypoxia Inducible Factor alpha; IFNγ - Interferon gamma; TNFα – Tumor Necrosis Factor Alpha)

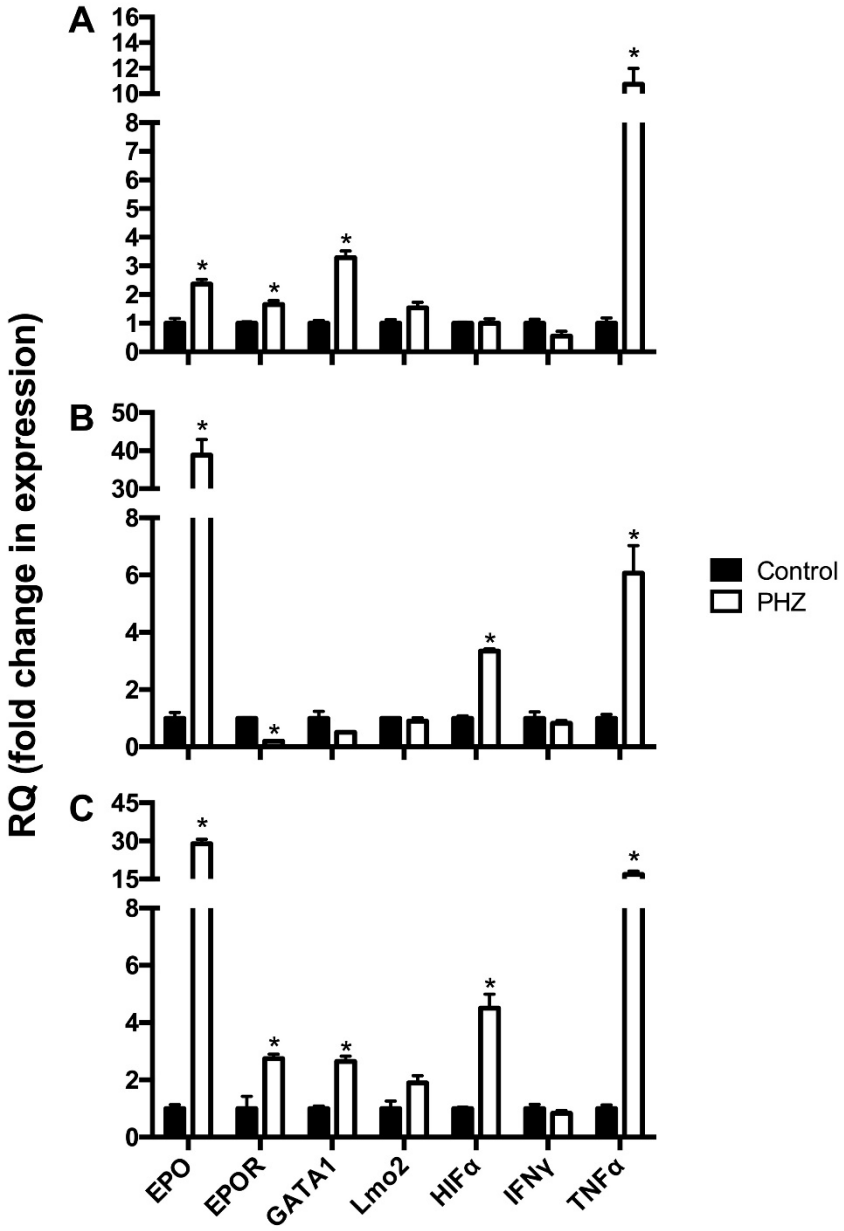

Figure 3.4 Kidney (A), liver (B) and spleen (C) mRNA levels of genes encoding erythropoiesis regulators 48 hrs post injection of PHZ to goldfish.
Expression was relative to endogenous control gene, EF-1α, and normalized against those observed in non-infected control fish for each gene. Data are represented as mean ± SEM of 4 fish ($P < 0.05$). Asterisks (*) denote statistical significance from non-infected control fish. (EPO – Erythropoietin; EPOR – Erythropoietin receptor; HIFα – Hypoxia Inducible Factor alpha; IFNγ - Interferon gamma; TNFα – Tumor Necrosis Factor Alpha)

CHAPTER 4

IMPACT OF RECOMBINANT ERYTHROPOIETIN ADMINISTRATION ON GOLDFISH (*Carassius auratus* L.) HEMATOLOGIC PARAMETERS DURING THE COURSE OF INFECTION WITH *Trypanosoma carassii*[1]

4.0 Introduction

Erythrocytes serve several essential functions in fish and can serve as a reliable indicator of teleost health and environment. Fish erythrocytes, like mammalian cells, contain hemoglobin which bind and transport oxygen throughout the body. These cells are also required for the transport and elimination of carbon dioxide and ammonia waste through the gills (55). Although standardized hematologic values have been established to serve as a baseline, erythrocyte numbers and morphology can fluctuate in response to water quality, stress, nutrition, temperature, and infection (128). The median value for total RBC counts is $1.8x10^9 \pm 0.2$ cells/mL, while PCV is generally measured to be 31.8 ± 5 percent (55) Decreases below the normal range is classified as anemia, which can have serious impacts on the overall health of the organism.

Due to the critical role that EPO plays in erythropoiesis, its regenerative effects have been a target of interest in the prevention and treatment of a variety of anemias. Recombinant EPO has been used to effectively treat patients suffering from anemia caused by renal disease and hormone-disrupting cancers (192). Additionally, treatment with

[1] A portion of this chapter has been published: McAllister M, Phillips N, Belosevic M. "*Trypanosoma carassii* infection in goldfish (*Carassius auratus* L.): changes in the expression of erythropoiesis and anemia regulatory genes" *Parasitology Research* vol. 118, issue 4, 1147-1158.

erythropoietin has been shown to be effective in reversing anemia caused by chemotherapy regimens (193). Parasitically induced anemia is a well-established phenomenon, and is a major pathology associated with infection in species such as *Plasmodium sp*. and *Trypanosoma sp.* (194,195). Administration of recombinant EPO in mice infected with *P. chabaudi* was shown to enhance recovery in B6 mice, and reduce the overall mortality associated with parasitic infection in A/J mice (196). Similar findings have been reported in mice infected with *P. berghei* (197). EPO treatments during the first 10 days of infection increased survival rate, and limited precipitous decreases in PCV observed in untreated mice infected with *T. congolense* (181).

Recombinant EPO has also demonstrated bioactivity when injected into non-mammalian models. Two species of teleost fish injected recombinant human EPO demonstrated significantly increased RBC counts and hemoglobin levels when compared to saline injected control groups. Previously in our lab, a recombinant goldfish EPO (rgEPO) molecule has been generated, which demonstrates *in vitro* activity by increasing proliferation, survival and differentiation of erythroid progenitor cells (122).

4.1 Methods

4.1.1 Infection of goldfish with *T. carassii*

Goldfish were infected with *T. carassii* as previously described in Chapter 3, Section 3.1.1. Following infection, blood was then collected on days 4, 7, 14, 28, 42 and 56 post infection from caudal vein using heparinized 25 GA needle and tuberculin syringe to examine intensity of infection. Following collection, blood was transferred to a 0.6mL microtube for further sampling. Parasitemia was measured by spinning a 70μL sample of

blood in a microhematocrit capillary tube at 16,000 x *g* for 5 minutes. The capillary tube was then filed at the RBC/serum interface and broken so that the serum containing the parasites could be diluted in PBS. The number of parasites were enumerated using a Neubauer hemocytometer, log transformed, and then presented as mean ± SEM parasites per mL of blood. Groups of 12 fish in replicate tanks were used for each of the treatments and control groups during cohort studies, with an *n*= 8-12 for each timepoint accounting for variations in the sample size because of either failure to obtain blood samples from all fish in the experimental group or host mortality in the high-dose group.

4.1.2 Production and purification of recombinant goldfish erythropoietin

Recombinant goldfish EPO (rgEPO) has been previously produced and purified as described (122). In brief, BL21 (DE3) *E. coli* cells transformed with an EPO insert in SUMO were grown and induced with 0.1 mM Isopropyl-β-D-L-thiogalactyopyranoside (IPTG). Cells were then lysed using 1X FastBreak Cell Lysis Reagent (Promega), and incubated with MagneHis Ni-particles (Promega). A magnetic column was then used to retain Ni-particles bound to His-tagged rgEPO. Supernatants were discarded, beads washed with wash buffer (100 mM Hepes, 500 mM NaCl, 20 mM imidazole, pH 7.5), and beads eluted using elution buffer (100 mM Hepes,500 mM NaCl, 500 mM imidazole, pH 7.5). His- and SUMO-tag were cleaved using SUMO protease (Invitrogen) and purified using MagneHis Ni-particles. Endotoxin traces were removed using Pierce High Capacity Endotoxin Columns (Thermo Scientific) according to manufacturer's directions. Protein samples were run on SDS-PAGE gels to determine purity, extracted, and confirmed as

goldfish EPO using mass spectrometry (Alberta Proteomics and Mass Spectrometry Facility) (Figure 4.1)

4.1.3 Administration of rgEPO

Goldfish were weighed, and average weight was used to determine injection volumes. Fish receiving EPO treatments were injected with 100ng/g of rgEPO in 0.9% saline solution on days 0, 4, 10, and 24. This was administered either alone (EPO group), or following infection with 1 x 10^7 parasites at day 0 (HD + EPO). The concentration of recombinant protein was selected based on preliminary studies which showed a modest effect on PCV. Fish infected with 1 x 10^7 parasites displayed a more severe anemia in preliminary studies and was therefore chosen to test physiological effects of rgEPO.

4.1.4 Assessment of hematologic parameters

Blood was collected from the caudal vein on days 4, 7, 14, 28, 42 and 56 post infection using a heparinized 25 GA needle and tuberculin syringe. Blood was then transferred from the syringe into a 0.6mL microtube so that samples could be taken for the following assays. Packed cell volume was determined by transferring a 70µL blood sample to a heparinized microhematocrit capillary tube (Fisher). Capillary tubes were then centrifuged at 10,000 rpm for 5 minutes, and the PCV was measured by dividing the volume of the packed erythrocytes by the total volume of the blood sample. Red-cell counts were performed as previously described (198). Briefly, 10µL of blood was serially diluted to a final concentration of 1:1000 in isotonic 0.9% saline solution and enumerated using a Neubauer hemocytometer. Hemoglobin levels were assessed using a Hemoglobin Assay kit (Sigma-Aldrich) as recommended by the manufacturer. A 2µL sample of blood diluted 1:100 in nuclease-free water (Ambion) was mixed with reagent and incubated for 5

minutes. Absorbance was then measured at 400nm using a SpectraMax M_2 microplate spectrophotometer (Molecular Devices). Mean erythrocyte volume (MEV) was derived from the hemoglobin values using the formula (198):

$$\boldsymbol{MCV = \frac{Hematocrit(\%) \times 10}{RBC\ (\times 10^{12}/L)}}.$$

4.1.5 Statistical analysis

Statistical analysis for blood metrics was performed using two-way analysis of variance (ANOVA) followed by a Holm-Sidak multiple comparison post-hoc test. A probability level of $P < 0.05$ was considered significant. Pearson r correlation analyses were used to establish *r* and R^2 values, with a probability level of $P < 0.001$ considered significant.

4.2 Results

4.2.1 *T. carassii* induces anemia during course of infection

Fish were injected with two different doses of *T. carassii* to determine whether the induction of anemia occurred in a dose dependent manner. In fish injected with 6.25 x 10^6 parasites (low dose), PCV and RBC counts decreased to a minimum at 14 dpi which was followed by a steady increase to above baseline levels at 56 dpi (Figure 4.2). Fish injected with 1 x 10^7 parasites (high dose) displayed a slightly different timeline leading to a more severe anemia, with PCV and RBC counts continuing to decrease until 28 dpi (Figure 4.2). Recovery was similar to that observed for low dose, with blood metrics returning back to above baseline levels 56 dpi. Number of parasites in the periphery was inversely related to PCV and RBCs during the course of infection, with peak parasitemia observed during peak

anemia for the high infection dose (Figure 4.3). Parasitemia was negatively correlation to both PCV and RBC counts, with a R^2 value of 0.5 and 0.43 respectively (Figure 4.4A-B).

4.2.2 rgEPO does not significantly change blood metrics in non-infected fish

EPO was administered at four different time points in non-infected control goldfish. Marginal increases were observed in both the PCV and total RBCs during the study, however these values were not statistically significant. PCV remained almost identical to control values following the first injection of EPO (36.9±0.9% and 36.6±0.7% for EPO and control groups respectively), but steadily increased following subsequent injections to a max of 40.9±1.0% and 41.0±1.0% on days 42 and 56 respectively (Figure 4.5A). RBC counts were slightly higher than control values following the initial injection of EPO and remained relatively constant throughout the observation period, with an overall average of $1.74 \times 10^9 \pm 0.05$ RBCs/mL and $1.80 \times 10^9 \pm 0.06$ RBCs/mL for control and EPO treatment groups, respectively (Figure 4.5B).

4.2.3 Administration of rgEPO alters progression of anemia and parasitemia in *T. carassii* infected goldfish

Administration of rgEPO lessened, but did not completely prevent, decreases in blood metrics during the acute stages of *T. carassii* infection. PCV and RBC counts remained static from 7 to 28 dpi in contrast to infected fish that did not receive EPO injections (Figure 4.6A-B). Following cessation of EPO injections, a sharp decrease in PCV and RBC numbers occurred at 42 dpi which was then followed by a slight recovery in PCV and RBC numbers at 56 dpi. Progression of parasitemia in fish receiving EPO injections

closely resembled that of an untreated infection from 4 to 14 dpi, however parasite numbers plateaued following day 14 with significantly fewer parasites ($3.41x10^7 \pm 1.6$ parasites/mL in EPO treated vs. $2.77x10^8 \pm 0.9$ parasites/mL in untreated) recorded at 28 dpi (Figure 4.7). The number of parasites subsequently spiked to a max of $3.45x10^8 \pm 2.6$ parasites/mL at day 42 before beginning to decline, similar to what was observed in untreated fish.

4.2.4 Concentration of hemoglobin in circulation decreases during infection

The concentration of hemoglobin in the circulation paralleled the changes in the blood metrics during the course of the infection. Fish infected with *T. carassii* demonstrated steadily decreasing levels of hemoglobin until 28 dpi, after which there was an increase to slightly above baseline levels (Figure 4.8A). Administration of EPO in non-infected control fish did not significantly change the concentration of hemoglobin, however, changes were observed in *T. carassii* infected fish that were injected with recombinant EPO. Hemoglobin levels remained steady from 4 to 28 dpi, however, a sharp decrease in hemoglobin concentration was observed at 42 dpi, followed by a return to baseline levels at 56 dpi.

4.2.5 Erythrocyte volume is not significantly altered during infection

Mean erythrocyte volume (MEV) describes the average size of RBCs, and can be used to infer the relative maturity of erythrocytes in circulation (185). The MEV for *T. carassii* infected fish remained relatively constant around 212fL throughout the infection, with a slight decrease observed at 56 dpi (Figure 4.8B). Uninfected fish treated with EPO had marginally increased MEV values from days 28 onward, however this increase was not

statistically significant. Administration of EPO to fish infected with *T. carassii* caused a spike in the MEV at 28 dpi, which subsequently decreased to starting values at 56 dpi.

4.3 Discussion

Infection of goldfish with *T. carassii* leads to pathophysiology similar to what has been observed in trypanosomes of higher vertebrates, with the key pathophysiological feature being anemia. *T. carassii* demonstrated a dose-dependent pathology, with a higher inoculation dose resulting in more severe anemia. In infection models using extracellular trypanosomes, development of anemia generally occurs in two distinct phases: acute and chronic. Acute phase is characterized by peak parasitemia and sharp decreases in circulating erythrocytes; whereas the chronic phase involves declining parasite numbers and a slow restoration of erythrocyte numbers (199). We observed a similar progression during the infection of goldfish with *T. carassii*, and correlation analysis revealed that decreases in PCV and total RBC counts were related to increasing parasitemia (Figures . 4.2A-B, 4.3). This inverse relationship suggests that the presence of the parasites acts as a trigger for the onset of anemia either directly or indirectly. The acute stage of anemia coincides with unchanged expression of erythropoietic regulator genes, whereas recovery is associated with significantly increased expression of most of these genes. Therefore, we examined whether exogenous administration of rgEPO was capable of compensating for this early suppression in the expression of erythropoiesis regulator genes that lead to anemia.

The administration of a recombinant *Xenopus laevis* EPO has been previously shown to stimulate proliferation of erythroid progenitors (200). Additionally, recombinant EPO has

also been shown to modulate anemia caused by parasitic infections. Mice infected with *Plasmodium chabaudi* display enhanced recovery and reduced mortality when injected with recombinant EPO (196). Similar findings have been reported for mice infected with *T. congolense*, where EPO treatments during the first 10 days of infection increased survival rate, and limited precipitous decreases in PCV observed in non-treated infected mice (181). Our results show that the administration of rgEPO reduces parasitemia and severity of anemia during the acute phase of infection. However, upon cessation of rgEPO injections there was a relapse characterized by increased parasitemia and decreased blood metrics with values similar to fish that received no rgEPO injections.

Hemoglobin (hb) is a primary hematological metric that is strongly correlated with PCV and total erythrocyte counts in goldfish and fluctuate in response to environmental factors, stress, nutrition and infection with pathogens (55). Peripheral hemoglobin levels decrease during hemolytic and nonregenerative anemia, which can arise as a result of parasitic infection in teleosts. Longhorn sculpin (*Myoxocephalus octodectemspinosus*) infected with *T. murmanensis* had hemoglobin levels that decreased simultaneously with PCV, which remained decreased for an extended period of time (201). Additionally, *Hypostoma spp.* infected with *Trypanosoma spp.* demonstrated decreases in hb that were correlated with parasite intensity (202). These findings have also been observed in mammalian systems, specifically in water buffalo infected with *T. evansi* (203). Our results corroborate previous findings, and show that the concentration of hb in goldfish is negatively correlated with parasite intensity. Although goldfish are known to be hypoxia-tolerant, this observed decrease in oxygen carrying capacity could be enough to signal

potentially kickstart erythropoiesis leading to the observed recovery from anemia (128,204).

To indirectly assess erythrocyte maturity, we determined mean erythrocyte volume (MEV). MEV values are indicative of the stage of development; with immature erythrocytes characterized by a smaller volume overall (204). Our results show marginal increases and decreases in MEV values in infected fish treated with rgEPO and those that were not, respectively. EPO has been shown to promote the survival of mature erythrocytes (205). This effect may work synergistically with the anti-inflammatory properties of EPO to protect both the progenitor and mature erythrocytes, helping to maintain static blood values during the acute phase of the infection. Alternatively, the observed plateau may also be the result of spatial limitations within the goldfish vasculature to accommodate erythrocytes, leukocytes and the parasites that require flagellar mobility in order to generate ATP and survive (206). This restriction on free space may slow parasite cytokinesis due to competition for space, therefore delaying pathogenesis associated with increased parasite burden and anemia as trypanosome motility has been shown to be essential for mediating pathogenesis (207).

Table 4.1 Parasitemia in goldfish infected with *T. carassii*

Days post-infection	No. of trypanosomes/mL (log_{10}) (Mean ± SEM)			
	LD	HD	p-value	
4	6.40 ± 0.60	6.79 ± 0.66	0.303	ns
7	6.54 ± 0.51	7.25 ± 0.67	0.029	ns
14	6.67 ± 0.61	7.54 ± 0.71	0.111	ns
28	5.67 ± 0.59	8.44 ± 0.79	0.011	*
42	4.97 ± 0.53	6.88 ± 0.65	0.111	ns
56	4.01 ± 0.46	5.95 ± 0.57	0.153	ns

Goldfish were inoculated with either 6.25 x 10^6 (LD) or 1 x 10^7 (HD) parasites. Microhematocrit capillary tubes were filed at the RBC/serum interface, and the serum including the parasites was diluted in PBS. Parasites were then enumerated using a Neubauer hemocytometer. Parasitemia is expressed as number of parasites/mL of blood, log transformed. Asterisk (*) denotes statistical significance ($p<0.05$) between LD and HD groups (n=8-12 fish per group).

Table 4.2 Parasitemia in goldfish infected with *T. carassii* following treatment with rgEPO

Days post-infection	No. of trypanosomes/mL (log_{10}) (Mean ± SEM)			
	HD	HD+EPO	p-value	
4	6.79 ± 0.66	6.83 ± 0.63	0.933	ns
7	7.25 ± 0.67	7.04 ± 0.62	0.394	ns
14	7.54 ± 0.71	7.57 ± 0.71	0.941	ns
28	8.44 ± 0.79	7.53 ± 0.67	0.024	*
42	6.88 ± 0.65	8.53 ± 0.81	0.165	ns
56	5.95 ± 0.57	7.12 ± 0.59	0.047	*

Fish were injected intraperitoneally with 1 x 10^7 parasites at day 0. rgEPO was administered intraperitoneally on days 0,4,10, and 24. Microhematocrit capillary tubes were filed at the RBC/serum interface, and the serum including the parasites was diluted in PBS. Parasites were then enumerated using a Neubauer hemocytometer. Parasitemia is expressed as number of parasites/mL of blood, log transformed. Asterisk (*) denotes statistical significance ($p<0.05$) between HD and HD+EPO groups (n=8-12 fish per group).

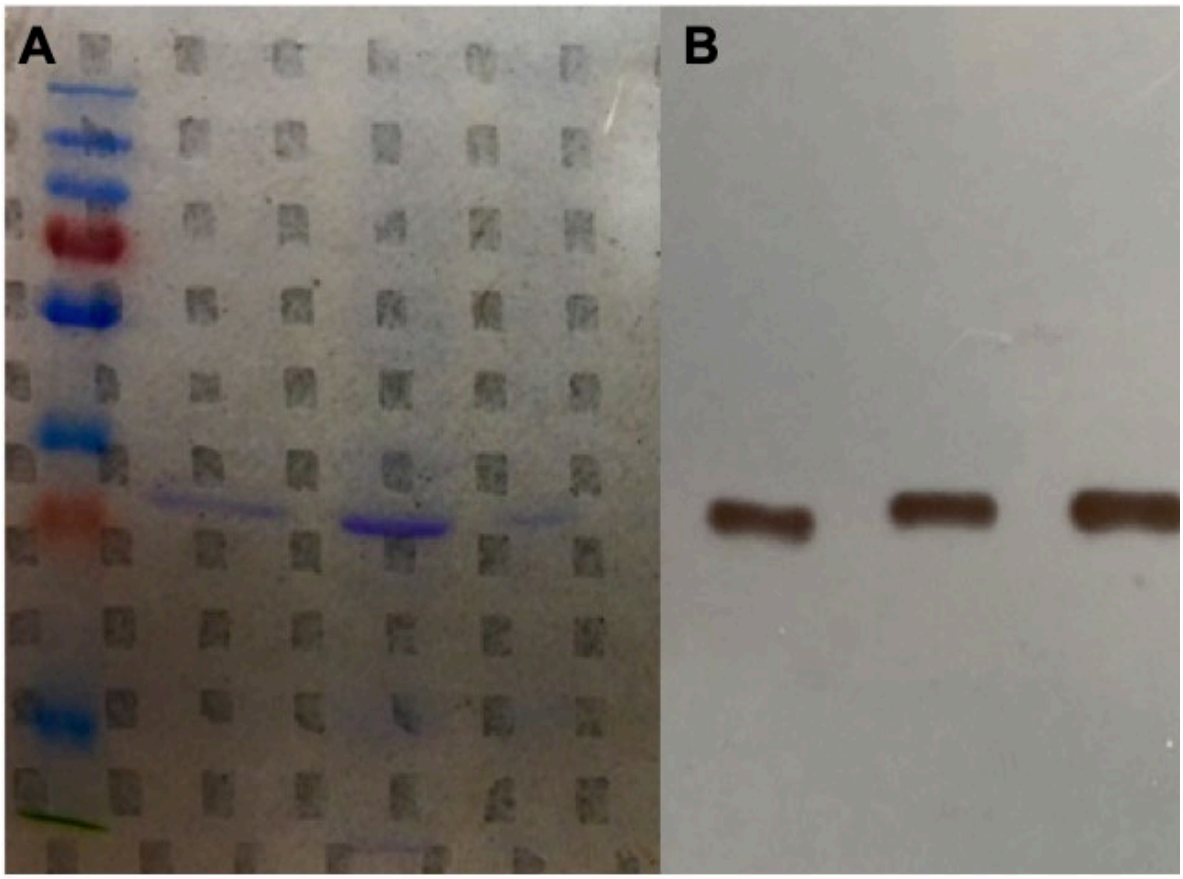

Figure 4.1 Recombinant expression of Erythropoietin (EPO).
Coomassie stain, showing protein bands at the expected MW of EPO + SUMO (~34kDa) (**A**). Western Blot using anti-6x His tag antibody with bands present at the same MW as Coomassie stain (**B**).

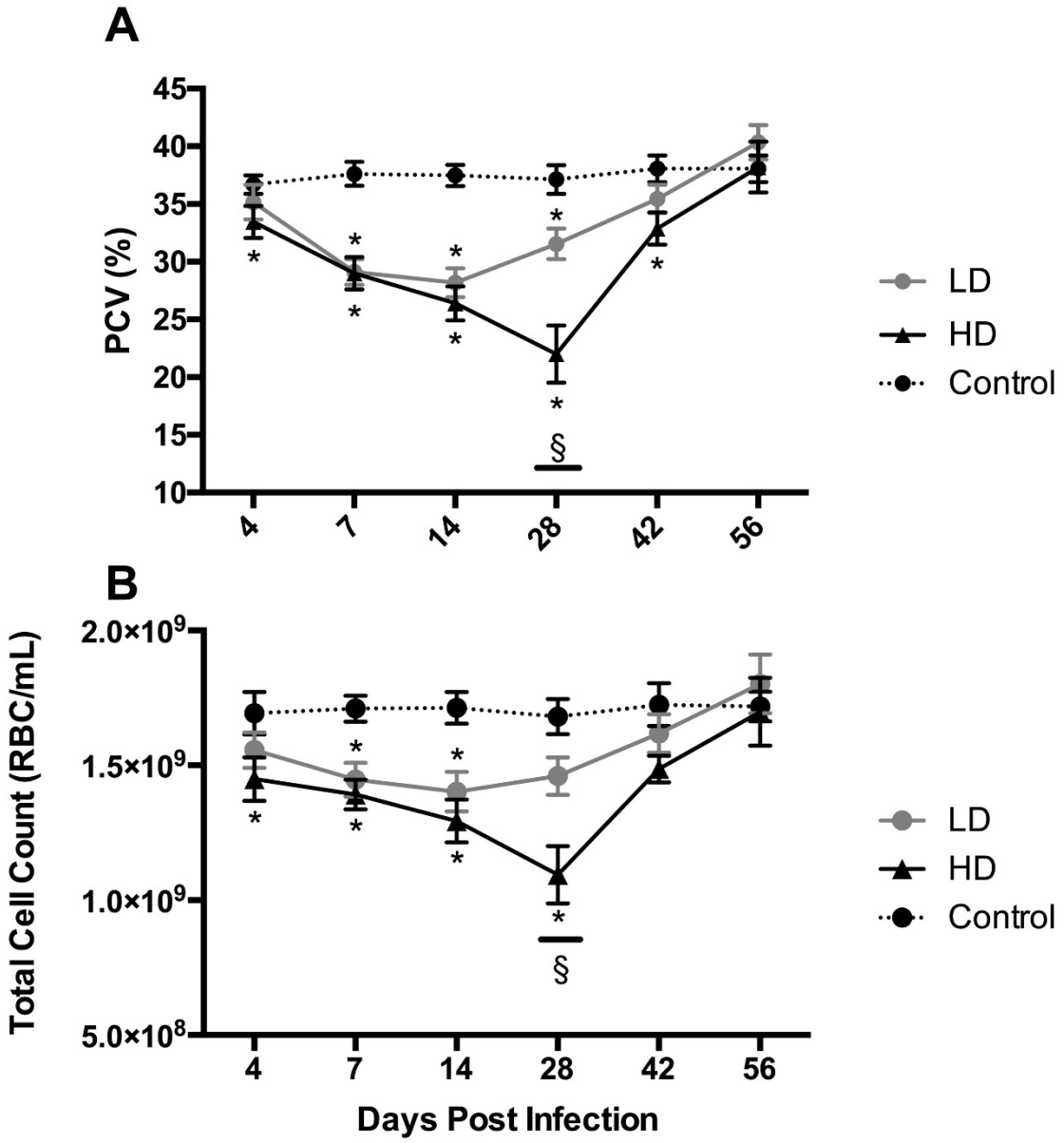

Figure 4.2 Decrease in blood metrics are dependent on parasite dose.
Treatment groups were inoculated with either 6.25 x 10^6 (LD) or 1 x 10^7 (HD) of *T. carassii*. Control groups were inoculated with TDL-15 medium. (A) Packed cell volume (PCV) is expressed as %RBC in total sample. Blood samples were collected at each day post infection, transferred to heparinized microhematocrit capillary tubes and spun at 10,000 rpm for 5 min. (B) Number of circulating red blood cells (RBCs), expressed as cells/mL of blood. Samples were serially diluted in PBS, and then enumerated using a Neubauer hemocytometer. Control groups across all days were averaged and presented as a dotted line. Asterisk (*) denotes statistical significance ($p<0.05$) from medium control. § denotes statistical significance ($p<0.05$) between treatment groups (n=8-12 fish per group).

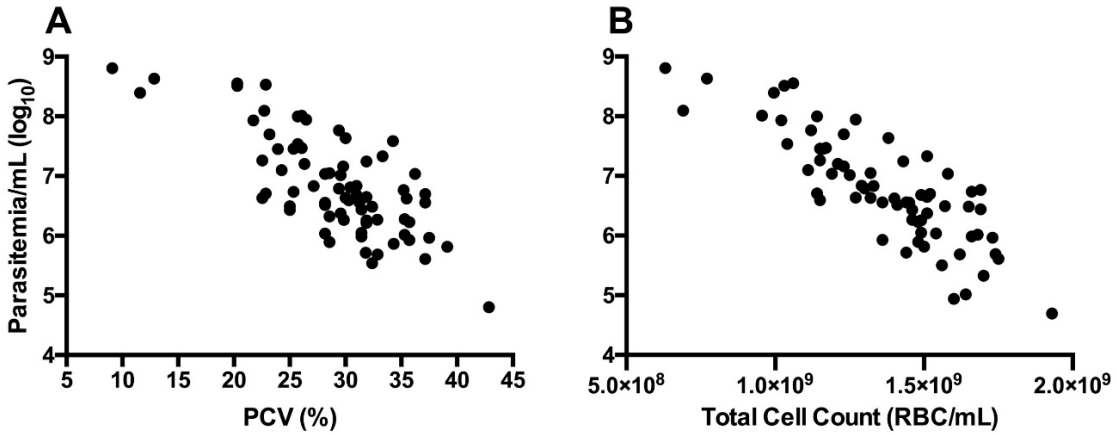

Figure 4.3 Parasitemia is related to both PCV values (A) and RBC numbers (B). Parasitemia and blood metric data from individual fish at each day post infection were matched with one another and correlational analysis was performed to determine correlational coefficient (r) and coefficient of determination (R^2). Values were determined to be statistically significant ($p<0.0001$). A: $r = -0.71$, $R^2 = 0.50$. B: $r = -0.65$, $R^2 = 0.43$

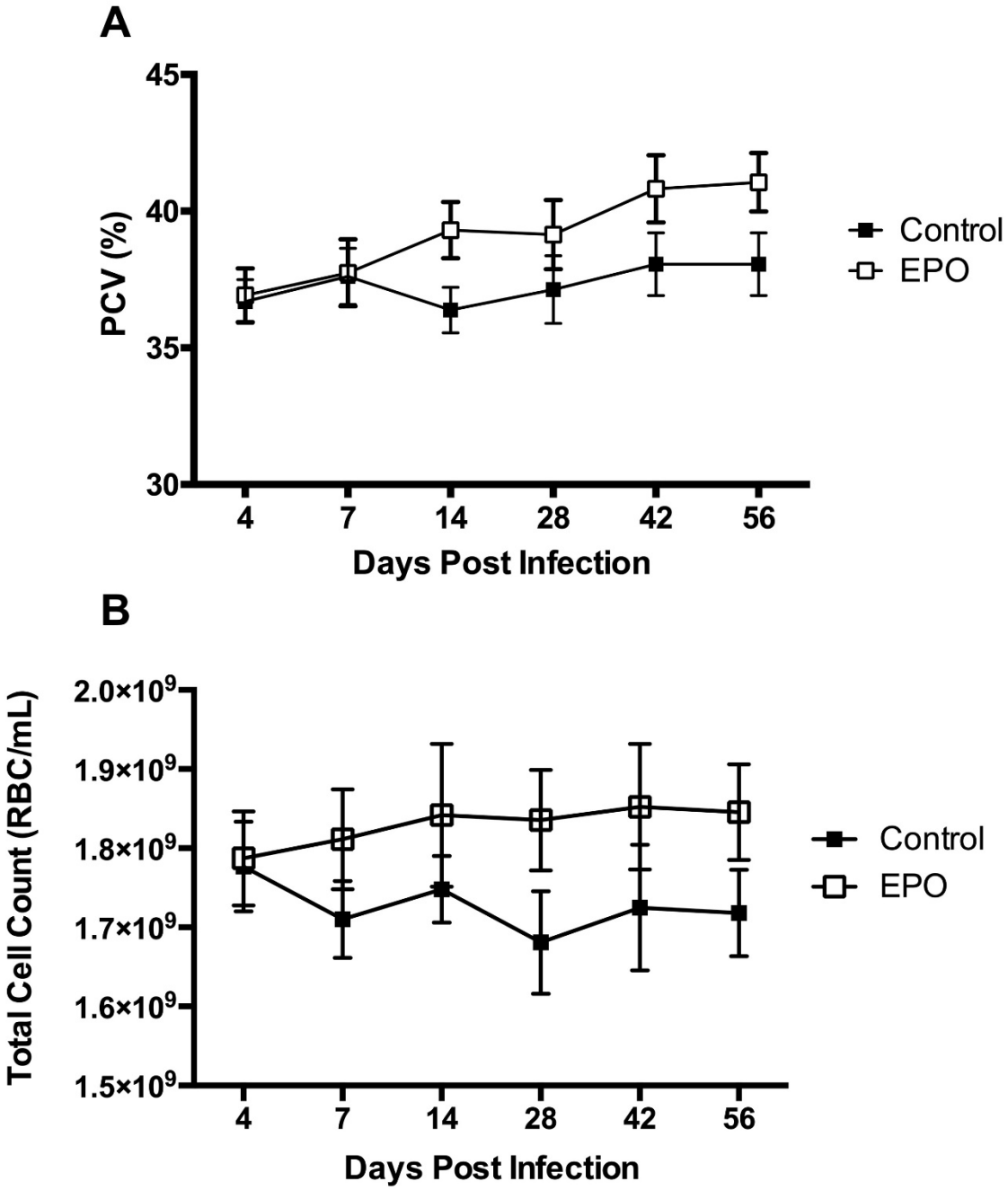

Figure 4.4 Administration of rgEPO does not significantly alter either PCV (A) or total RBC counts (B) during infection with *T. carassii*.
EPO treatments were injected with 100ng/g of rgEPO in 0.9% saline solution on days 0, 4, 10, and 24. Control groups were inoculated with TDL-15 medium (n=8-12 fish per group).

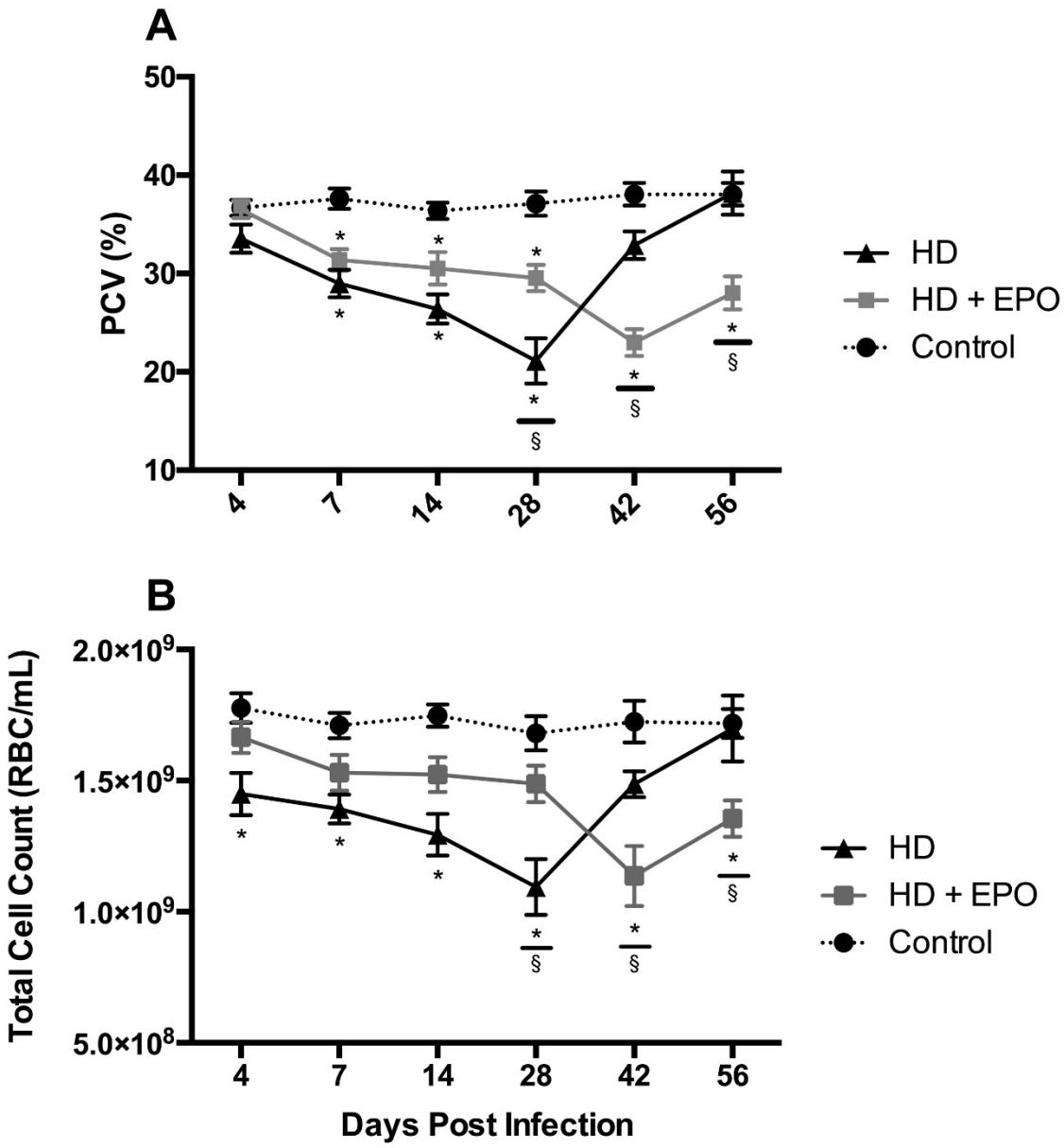

Figure 4.5 Recombinant goldfish erythropoietin (rgEPO) injections delay the onset of anemia in fish infected with *T. carassii.*
Treatment fish were injected with either 1 x 10^7 parasites at day 0 or were injected with both parasites and EPO together (HD + EPO group). The HD + EPO group received 100ng/g of rgEPO at days 0, 4, 10, and 24. Control groups were sham injected with TDL-15 medium. (**A**) PCV is expressed as %RBC in total sample. (**B**) Number of circulating RBCs, expressed as cells/mL of blood. Control values were averaged across all days, and presented as a dotted line. Asterisk (*) denotes statistical significance ($p<0.05$) from medium control. § denotes statistical significance ($p<0.05$) between treatment groups (n=8-12 fish per group)

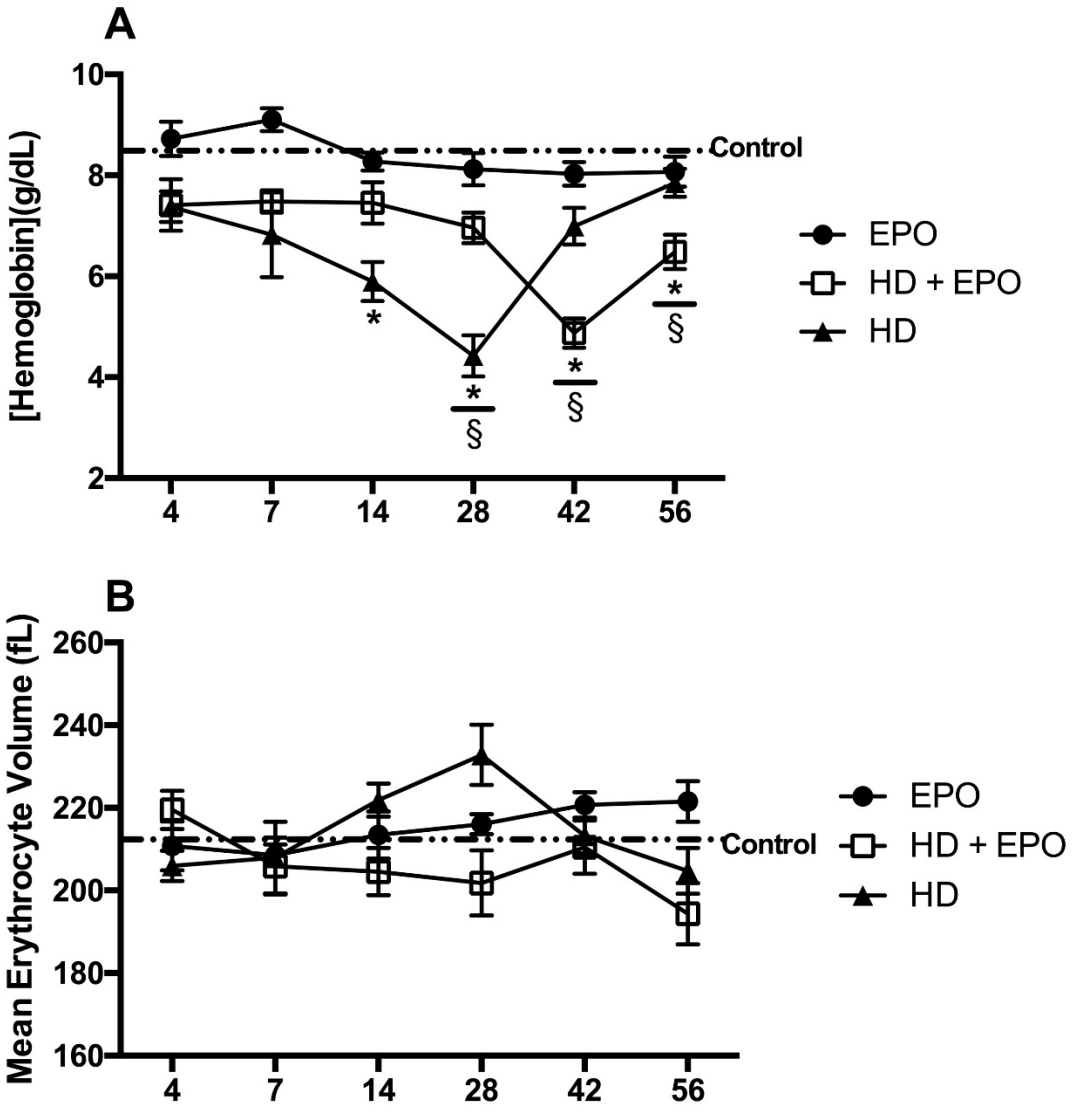

Figure 4.6 Infection of goldfish with *T. carassii* causes a decrease in hemoglobin with no significant changes in mean erythrocyte volume (MEV).
(**A**) Hemoglobin values were determined by colorimetric change at 400nm using a Hemoglobin Assay Kit (Sigma-Aldrich). (**B**) MEV was determined using a standard formula described in section 4.1.4 of Methods. Asterisk (*) denotes statistical significance ($p<0.05$) from medium control. § denotes statistical significance ($p<0.05$) between treatment groups (n=8 to 12 fish per group).

CHAPTER 5

IMPACT OF GOLDFISH (*Carassius auratus* L.) AND *Trypanosoma carassii* FACTORS ON ERYTHROID DEVELOPMENT AND SURVIVAL

5.0 Introduction

Erythrocyte development is a highly conserved process between mammals and fish. Impairment in the development and maintenance of erythroid populations can be detrimental to the organism, and therefore needs to be tightly regulated to ensure that the organism has a sufficient cell pool to meet metabolic demands and maintain homeostasis. Due to the critical role that these cells play in the organism, examination of this process has been an area of significant interest. Colony-forming assays have been used to visualize and monitor the development of hematopoietic stem cells (HSCs) along different lineages after stimulation with various growth factors. These assays utilize semi-solid mediums to promote progenitor cells division and differentiation into distinct colonies (208). Development of erythroid colonies arising from HSCs has been well documented and follows a defined sequence previously described in my Literature Review (Chapter 2). The influence of activating and inhibitory stimuli can then be directly observed via changes in the number, and make-up of these colonies.

Proinflammatory cytokines have been previously shown to negatively influence hematopoiesis, and specifically erythropoiesis. IFNγ was shown to induce apoptosis in mammalian erythroid progenitor cells, and has also been reported to exert suppressive effects on cell growth (209). TNFα has been implicated in impairing normal erythrocyte development by suppressing progenitor cell differentiation (113). Additionally, high

mobility group box protein 1 (HMGB1) has been shown to be a potent inhibitor of erythropoiesis *in vitro*. The addition of exogenous HMGB1 to methylcellulose cultures of HSCs lead to significant reductions in erythroid cell proliferation as well as increased apoptosis of erythroblasts (210).

In addition to targeting the developmental stages, mature erythrocytes serve as targets for direct cytotoxic effects mediated by pathogens such as *Trypanosoma* spp. Parasite derived factors have been shown to directly mediate extravascular hemolysis (211). *Trypanoplasma borrelia* has been shown to produce a factor which directly leads to erythrocyte lysis (78). Additionally, *T. cruzi* has been shown to produce a hemolysin molecule which causes pore formation in erythrocytes, leading to membrane degradation and cell death (94).

In this chapter I report on the *in vitro* effects of recombinant proinflammatory cytokine administration on erythroid progenitor development, and potential direct hemolytic effects of *T. carassii* derived factors on goldfish erythrocytes.

5.1 Methods

5.1.1 Isolation of primary kidney macrophages (PKM)

Isolation of PKM cells was performed as previously described (137). Goldfish were anesthetized in TMS-222, and the kidney was asceptically removed and placed in homogenizing solution (MGFL-15, 100U/mL penicillin/100µg/mL streptomycin, and 50U/mL heparin). The kidneys were then passed through steel mesh screens and washed with homogenizing solution. The cells were then layered on a 51% Percoll-media gradient and centrifuged at 400 x *g* for 25 minutes. Cells at the gradient interface were then

extracted and washed twice in MGFL-15 (230 x *g* for 10 minutes). Following the final wash, cells were immediately resuspended in FACS Sorting Buffer (1X PBS, 1% fetal bovine serum, 4mM EDTA).

Early progenitor cells were gated using previously defined parameters (212). Freshly isolated cells were sorted based on size and internal complexity using a FACS Aria flow cytometer, and then sorted into modified NMGFL-15 medium containing 10% calf serum, 1% carp serum, 100 U/mL of penicillin/100 μg/mL of streptomycin, and 100 μg/mL of gentamicin. Cells were then washed and cultured overnight at 20°C to recover.

5.1.2 Purification of recombinant tumor necrosis factor-alpha (TNFα) and interferon-γ (IFNγ)

Recombinant TNFα and IFNγ has been previously produced and purified as described (213,214). In brief, BL21 Star One Shot *E. coli* cells (Invitrogen) were transformed with the gene insert in a SUMO vector were grown for 2.5 hours, and then induced with 1mM of Isopropyl-β-D-L-thiogalactyopyranoside (IPTG) for 5 hours. Cells were then pelleted by centrifugation, and frozed overnight at -20°C. Bacterial pellets were lysed in 1X Fastbreak Cell Lysis Buffer (Promega) diluted in denaturing wash buffer (100mM HEPES, 10mM imidazole, and 7M urea, pH 7.5) and subsequently incubated with MagneHis Ni-particles (Promega). A magnetic column was used to retain Ni-particles bound to His-tagged IFNγ and TNFα. Supernatants were discarded, and beads were washed 4X under denaturing conditions. Beads were then eluted using denaturing elution buffer (100 mM Hepes, 500 mM imidazole, 7M urea, pH 7.5). Proteins were renatured overnight in 10 volumes of renaturation buffer (4mM reduced glutathione, 2mM oxidized glutathiones, 50mM soidium

borate, 5mM EDTA, pH 8.5 for rgIFNγ; 40mM Tris, pH 8.0 for rgTNFα), and then dialyzed overnight against 1X sterile PBS in 20, 0000 MWCO Slide-A-Lyzer dialysis cassettes (Thermo-Fisher). Presence of protein was verified using Commassie stain and Western blot analysis (Figure 5.1-2, A-B). Endotoxin traces were removed using Pierce High Capacity Endotoxin Columns (Thermo Scientific) according to manufacturer's directions. Protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce).

5.1.3 Methylcellulose colony assay

Methylcellulose stock was prepared as previously described (122). Briefly, 4% methylcellulose stock solution was prepared by adding 8g of methylcellulose (4000 cps, Sigma) to 200mL of Milli-Q water at 80°C. This solution was then autoclaved, cooled to room temperature and then left to stir overnight at 4°C to re-dissolve. The next day, 200mL of 2X MGFL-15 was added to the methylcellulose solution to create a final mixture of 2% methylcellulose in 1X MGFL-15. This mixture was stirred overnight at 4°C, and then stored at -20°C until use.

This assay was performed as previously described (122). R1 gated PKM cells isolated by FACS flow cytometry were resuspended to a final concentration of $3.3x10^5$ cells/mL in complete MGFL-15 (MGFL-15 medium additionally containing 20% calf serum, 2% carp serum, 200 U/mL of penicillin/200 μg/mL of streptomycin, and 200 μg/mL of gentamicin). One and a half mL of cell suspensions were then added to 2% methylcellulose medium at a 1:1 ratio, to create a 1% complete methylcellulose medium containing $5x10^4$ R1-gated progenitor cells.

Known amounts of the recombinant cytokines were added, and the methylcellulose medium was slowly mixed using a 3mL syringe with a 16-gauge needle to avoid the introduction of air bubbles. One mL of the solution was added in triplicate to each well of a non-tissue culture treated 6-well plate, sealed with parafilm, and incubated at 20°C for 10 days. Formation of erythroid colonies was assessed by microscopic examination of colony morphology and documented by photo-microscopy.

5.1.4 Collection of parasite supernatant

Parasite cultures of *Trypanosoma carassii* were maintained as glycerol stocks in our lab at -80°C until needed for assays. Thawed stocks were then maintained *in vitro* by serial passage through TDL-15 medium supplemented with 10% heat-inactivated (HI) goldfish serum, as previously described (7). Parasite cultures were incubated at 20°C and passed every 6-7 days. Parasite supernatant was isolated by centrifuging parasites 400 x *g* for 10 minutes, and then decanting into a separate tube. The supernatant was then filtered through a 0.22μm Millipore Express PES membrane (Millex) to remove any remaining parasites. Heat-inactivated supernatant was incubated in a water bath at 60°C for 1hr.

5.1.5 Erythrocyte Lysis Assay

Goldfish blood was collected from the caudal vein using a heparinized 23-gauge needle and 3mL Luer-Lock syringe. Samples were immediately transferred from the syringe into a 1.5mL microtube containing 10% trisodium-citrate and inverted 3 to 5 times to prevent coagulation. Subsamples of the blood were taken and added to separate tubes for each timepoint, followed by the addition of either *T. carassii* [heat-inactivated (HI) and non-HI] supernatant, physiological saline (0.9% NaCl), or ACK Lysing Buffer (Gibco) at a

1:1 v/v ratio. ACK buffer specifically targets red blood cells for lysis by disrupting the ammonia/ammonium equilibrium across the cell membrane, causing RBCs to rupture (215). Separate tubes for each timepoint were used to ensure that no lysis was caused by pipetting or centrifugation. Individual treatment groups were mixed by inversion in a 1.5mL microtube, and then a sample was taken to act as a baseline value. The mixtures were then incubated for 3, 6, and 18 hours at 20°C. At each timepoint, blood samples were centrifuged at 1,600 x *g* for 15 minutes. Serum was collected following centrifugation, and the amount of hemoglobin was measured as a proxy for erythrocyte lysis. Hemoglobin levels were measured as previously described in Chapter 4, Section 4.1.4.

5.1.6 Statistical Analysis

Colony formation and hemoglobin release assays were analyzed using unpaired multiple *t*-test, followed by a Holm-Sidak multiple comparison post-hoc test. A probability level of $P < 0.05$ was considered significant.

5.2 Results

5.2.1 rgTNFα and rgIFNγ impair erythroid colony formation

R1-gated progenitor cells incubated without the addition of EPO did not form any erythroid colonies (Figure 5.3A). When EPO was added at a concentration of 50ng/mL, based on previous studies which demonstrated maximum *in vitro* effects on colony formation (122), colony formation was observed (Figure 5.3B). Colonies were composed of tightly aggregated cells that were spherical in appearance (Figure 5.3C), similar to what has been described in both mammalian and teleost colony formation (122,216). When rgTNFα

and rgIFNγ were added to cell cultures in addition to EPO, colonies were still detected that resembled those seen in the EPO control wells (Figure 5.3D-F). However, upon addition of proinflammatory cytokines, areas of clearance began to form in the methylcellulose medium. This phenomenon was most pronounced in wells containing 100ng/mL of both IFNγ and TNFα together with EPO. Cells were observed to clump together and form loose aggregates, which resembled diffuse cell colonies distinct from the erythroid colonies (Figure 5.3F, inset).

The addition of rgIFNγ and/or rgTNFα significantly reduced the number of erythroid colonies observed at all doses tested (Figure 5.4A-C). Number of colonies was reduced compared to EPO control wells in a dose-dependent manner, where cytokine concentrations of 100ng/mL caused the most significant reduction in erythroid colony numbers. Interestingly, both the 10ng/mL and 100ng/mL treatments of rgIFNγ+rgTNFα together lead to greater decreases in erythroid colonies compared to those induced by either rgTNFα or rgIFNγ at a dose of 100ng/mL (Figure 5.4C).

5.2.2 *T. carassii* culture supernatant increases release of hemoglobin from goldfish erythrocytes *in vitro*

Freshly isolated goldfish erythrocytes were incubated with supernatant collected from *in vitro* cultures of *T. carassii* to determine whether parasite-derived products caused release of hemoglobin into the serum. Physiological saline (0.9% NaCl) is an isotonic solution and was used as a control for spontaneous hemolysis over time. Incubation of erythrocytes with saline lead to small increases in serum hemoglobin (Figure 5.5A). Both non-heat inactivated and heat inactivated *T. carassii* supernatant caused to increases in

serum hemoglobin, with the greatest increases observed at 18hrs post incubation (Figure 5.5A). Heat-inactivation of the supernatant lead to small, but insignificant, decrease in the amount of hemoglobin released during incubation relative to non-HI supernatant. ACK buffer caused significantly more hemoglobin to be released into serum at all time points (Figure 5.5A).

The relative lysis of erythrocytes *in vitro* was determined to account for the effects of background hemoglobin release over time. Saline treatment was set as the reference, and experimental values were normalized against saline values at each time point. When controlling for time effect of spontaneous erythrocyte lysis, the amount of hemoglobin released into the serum during incubation with *T. carassii* supernatants remained higher than saline controls (Figure 5.5B). The measured hemoglobin release at 18 hours post-incubation for both non-heat inactivated and heat inactivated supernatant remained 10-fold lower than the ACK Lysis Buffer group (Figure 5.5B).

5.3 Discussion

In this chapter I report on the *in vitro* effects of proinflammatory cytokines on erythroid colony development. The colony forming assay is a standard for observing the process of erythropoiesis and has been used to study disease processes due to abnormal hematopoiesis in mammalian models. This assay was optimized previously in our laboratory using goldfish cells, and colony formation was shown to progress in a manner similar to what has been observed for mammalian cells. Both TNFα and IFNγ have been shown to negatively regulate erythropoiesis. These inhibitory effects are mediated at the progenitor cell stage, through altered gene expression and induction of apoptosis. The fact

that these cytokines are both upregulated during infection of goldfish with *T. carassii*, specifically during periods of anemia, suggests that these cytokines may affect the production of new erythrocytes (111). Therefore, I decided to examine whether the addition of rgIFNγ and rgTNFα altered the development of goldfish erythroid colonies using *in vitro* methylcellulose assay. My findings reveal that the addition of either rgIFNγ or rgTNFα, as well as rgIFNγ+rgTNFα in combination, significantly reduced the number of erythroid colonies, suggesting that these cytokines downregulated erythropoiesis.

My results showed that there was a dose-dependent reduction in erythroid colony formation after treatment with either 10ng/mL or 100ng/mL of rgIFNγ and/or rgTNFα. This reduction was not reversed by the addition of 50ng/mL of EPO, which has been shown to be the optimal concentration for erythroid colony formation *in vitro* (122). These results are similar to published findings that reported that both TNFα and IFNγ significantly reduced the number of erythroid colonies *in vitro*. TNFα was shown to be a highly potent inhibitor of erythroid colony formation of human bone marrow cells, and complete inhibition of colony-forming ability was observed at as little as 2ng/mL (217). The addition of EPO and stem cell factor (SCF) suppressed the inhibitory activity of TNFα, with maximal inhibition occurring at a concentration of 200ng/mL. Similar findings have been reported for mouse progenitor cells stimulated with IFNγ. Colony formation decreased in a dose-dependent manner with maximum inhibition occurring at 2μg/mL of rmIFNγ (218).

Both cytokines reduced the number of erythroid colonies. This suggests that rgIFNγ- mediated inhibition was not dependent on the presence of rgTNFα, and vice-versa. However, when both rgIFNγ and rgTNFα were added in combination there appeared to be

a additive effect as the number of colonies was lower for both doses than either rgIFNγ or rgTNFα alone (Figure 5.4C). Synergism and additive effects between proinflammatory cytokines is a well-established phenomenon and is a key mechanism in effective immune responses. TNFα is capable of enhancing cytotoxic activities of IFNγ causing oligodendrocyte progenitor cells death (219). They have also been shown to act together to downregulate cellular proliferation and differentiation (220). In erythroid colony assays, complex interactions between proinflammatory cytokines have been identified. Interleukin-1α (IL-1α) is capable of inhibiting colony formation *in vitro*, but relies on the presence of IFNγ to carry out these effects (221). Additionally, IL-1α has been shown to act synergistically with TNFα to suppress murine erythroid colony formation. The combination of sub-inhibitory doses (0.2ng/mL) of IL-1α and TNFα caused drastic decreases in the number of colonies observed (222)

Cell morphology was noticeably altered after the addition of rgIFNγ and/or rgTNFα, with cells appearing to clump together and form areas of clearance in the medium. Further examination revealed what appeared to be colonies composed of loosely aggregated cells, significantly less compact than the erythroid colonies induced by addition of EPO (Figure 5.3F). Zebrafish progenitor cells cultured in granulocyte-colony stimulating factor (G-CSF) produced similar spread colonies that possessed a ruffled appearance morphologically distinct from erythroid colonies, and were primarily composed of myeloid progenitor cells, as well as mature neutrophils and monocytes (223). These loosely aggregated colonies have also been identified in mouse and human colony assays, with the colonies being primarily composed of granulocytic precursor cells (208,224). The colony-like clumping of cells in the rgIFNγ+rgTNFα treatment could potentially be myeloid

colonies which were induced following excessive cytokine stimulation. Proinflammatory cytokines have been shown to promote differentiation of progenitor cells into the myeloid lineage. Erythroid precursor cells express receptors capable of binding TNFα and IFNγ, which then utilize a redundant signalling pathway to promote myeloid differentiation (225). Stimulation of these progenitor cells with cytokines that utilize this redundant pathway may lead to "signal hijacking", which activates an alternative subset of genes and drives differentiation away from the erythroid lineage.

In this chapter I also presented results of the hemolytic activity property of parasite supernatants collected from *in vitro* cultured *T. carassii*. Goldfish erythrocytes contain hemoglobin which can be released into solution when the integrity of the cell membrane is compromised. Measuring the amount of hemoglobin released into solution can be used to indirectly assess hemolysis of RBCs (226). Previous studies examining *T. carassii*-mediated anemia in goldfish have shown that incubation of erythrocytes with both live and sonicated parasites lead to lysis of RBCs and increases in the amount of hemoglobin released (23). This was thought to be mediated by a hemolysin produced by the parasite, capable of causing extravascular hemolysis. Live parasites were suggested to be able to cause mechanical damage to erythrocytes through flagellar motion, which could contribute to the observed increases in RBC lysis and hemoglobin release (211). Additionally, trypanosome autolysis has been shown to produce phospholipase A1 and lysophospholipase A1 which can cause membrane degradation and contribute to destruction of erythrocytes *in vitro* (227). Parasite sonification could lead to massive amounts of these fatty acids being released, which would be significantly higher than levels that would be released *in vivo*.

I was interested in determining whether parasites produced excretory/secretory (ES) factors during growth and replication, as opposed to releasing intracellular constituents upon death. My findings suggest that parasite supernatant may contribute to lysis of goldfish erythrocytes during coincubation (Figure 5.4, 5.5). The hemoglobin release followed a similar trend to what has been previously been described, with the amount of free hemoglobin in the serum increasing with incubation time. Although I tried to address the issues with some of the previous assays examining erythrocyte lysis, there are still several confounding factors which could also be contributing to the observed hemolysis. *In vitro* growth and replication of *T. carassii* leads to the production of a range of metabolites and active chemical substances which can be toxic to cells. Free-fatty acids, pyruvates, and aromatic by-products are all products produced by trypanosome growth which can have detrimental effects on erythrocyte membranes. Severe infections with *T. carassii* can lead to parasitemia levels that can approach a 1:1 ratio of parasites:RBCs. During these situations, an excessive amount of parasitic by-products would be generated that could lead to osmotic fragility and directly contribute to hemolysis of RBCs (211). However, the negative effects of these metabolites would be mitigated *in vivo* by filtration through the teleost renal system and excretion of waste products through the gills or urine (228). Additionally, the release of hemoglobin from erythrocytes leads to an increase in iron (Fe^{3+}) which has been shown to be toxic to cells at high concentrations (229). *In vivo*, iron levels in the serum are tightly regulated and free iron is quickly bound by a variety of iron transporters such as hepcidin, transferrin and hemopexin for recycling , storage, and sequestration (137). Because these mediators are not present *in vitro,* this may lead to a positive feedback loop of hemolysis as more and more iron is released from damaged

erythrocytes. Despite these limitations, the production of parasite by-products may also contribute to the anemia observed during infection of goldfish with *T. carassii*.

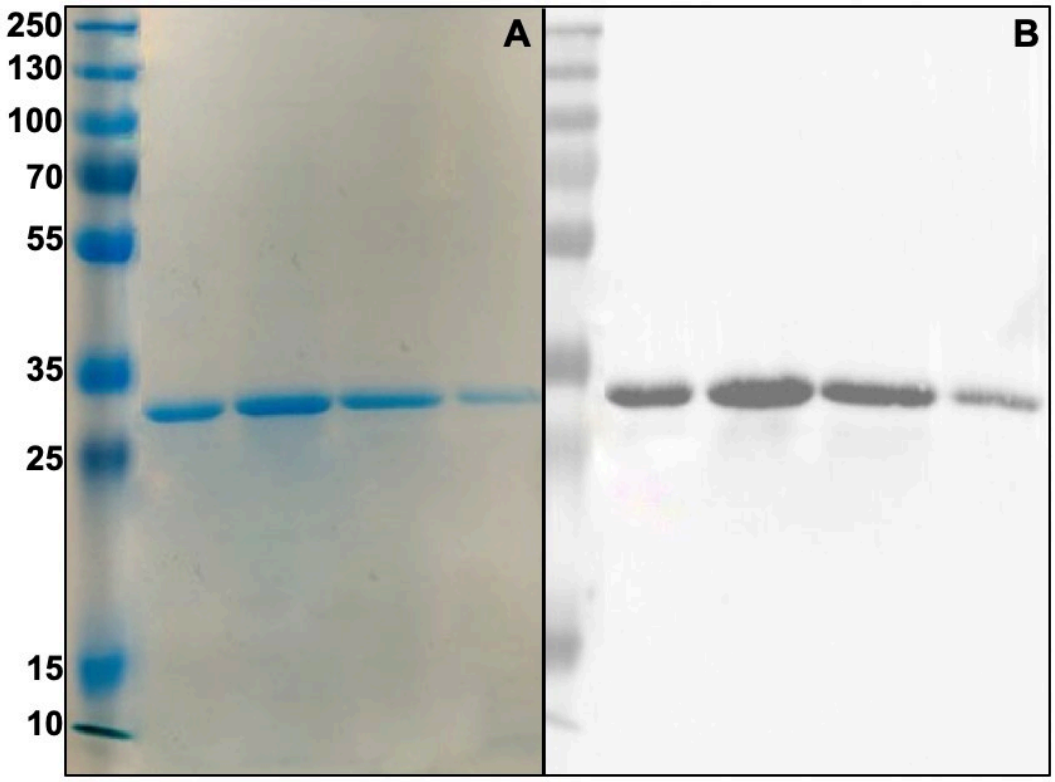

Figure 5.1 Recombinant goldfish interferon-γ (rgIFNγ).
Each band represents an individual elution. (A) Coomassie stain following reducing SDS-PAGE showing bands at the expected MW of IFNγ+SUMO. (B) Western Blot visualized using anti-6x His tag HRP-conjugated antibody (Abcam) with bands present at the same MW as seem in the Coomassie.

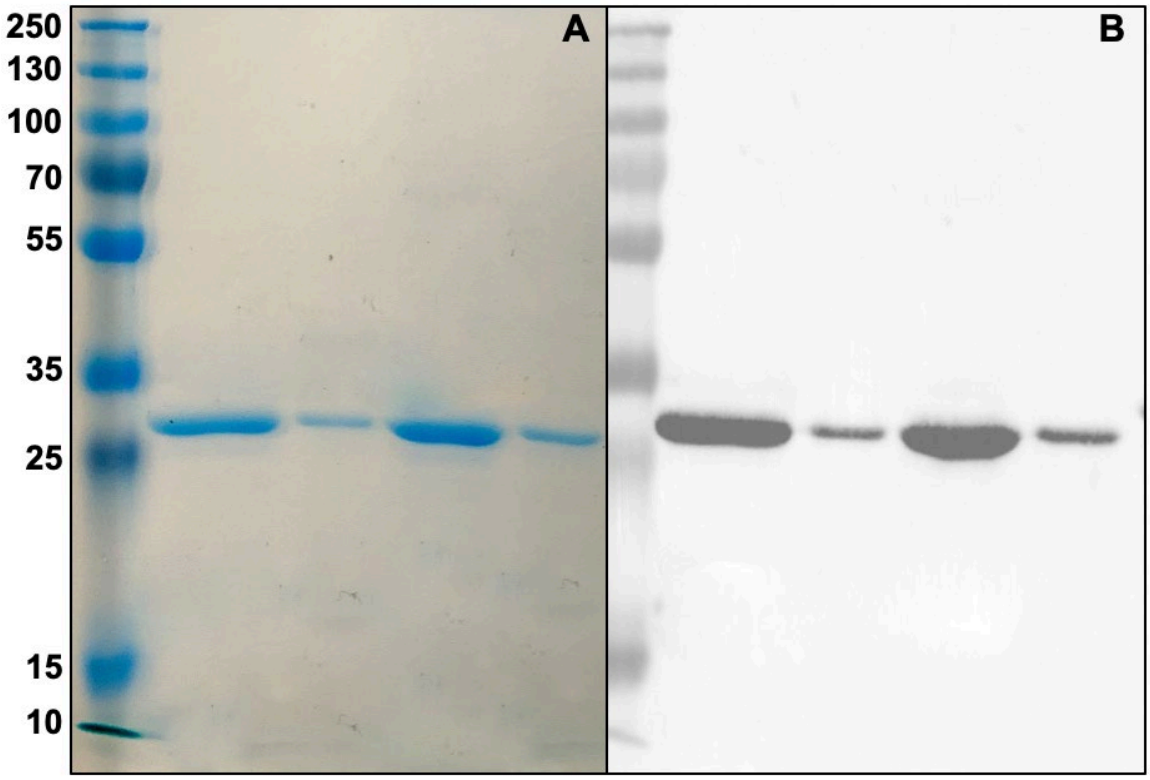

Figure 5.2 Recombinant goldfish tumor necrosis factor-α (rgTNFα).
Each band represents an individual elution. (A) Coomassie stain following reducing SDS-PAGE showing bands at the expected MW of TNFα+SUMO. (B) Western Blot visualized using anti-6x His tag HRP-conjugated antibody (Abcam) with bands present at the same MW as seem in the Coomassie.

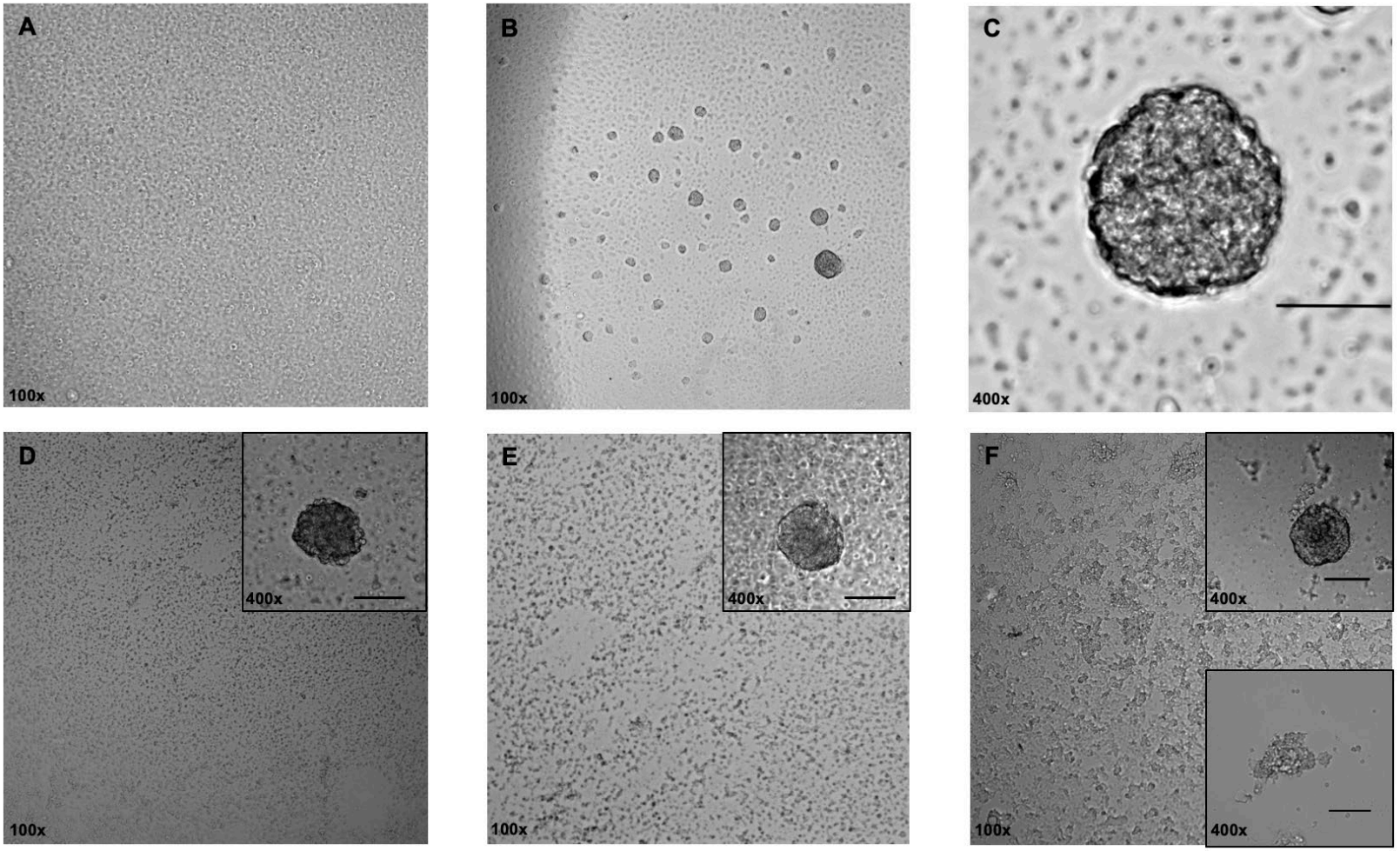

Figure 5.3 Photomicrographs of colonies in methylcellulose cultures in the presence and absence of EPO.
R1-gated progenitor cells were plated into the wells of a non-tissue culture treated 6-well plate at a concentration of $5x10^4$. Experimental groups received 50ng/mL of EPO in combination with either 10ng/mL or 100ng/mL of TNFα, IFNγ, or TNFα+IFNγ. (A) No treatment. (B-C) EPO alone at 100x and 400x magnification, respectively. (D) EPO + 100ng/mL of TNFα. (E) EPO + 100ng/mL of IFNγ. (F) EPO + 100ng/mL of IFNγ + 100ng/mL of TNFα.

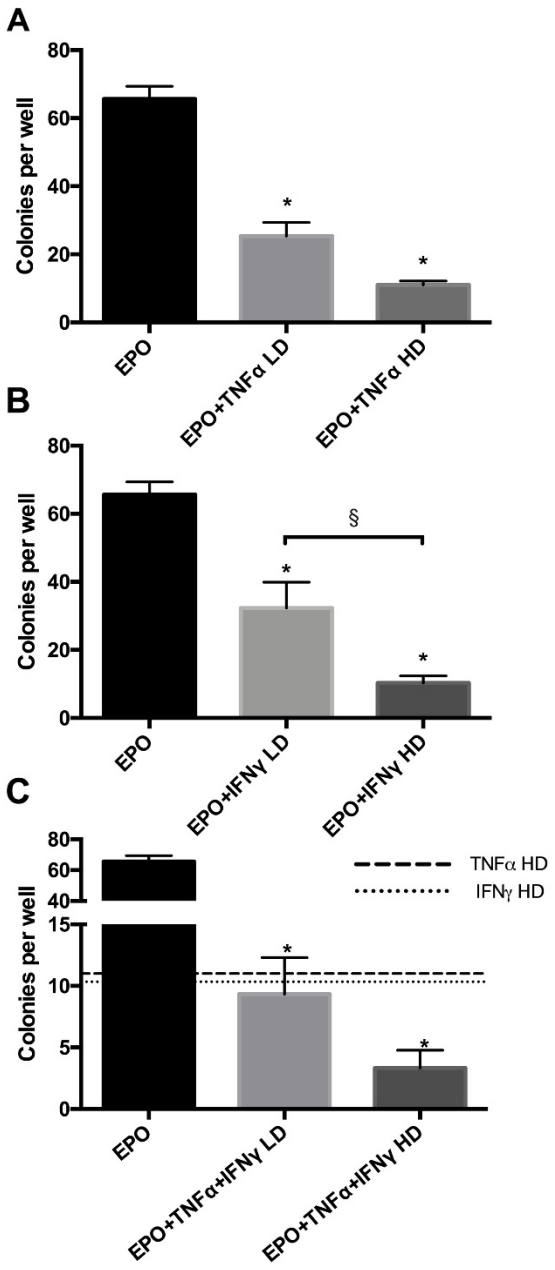

Figure 5.4 Erythroid colony formation per $5x10^4$ cells in complete methylcellulose medium following stimulation with recombinant proteins.
Cells were incubated at 20°C for 10 days, counted based on morphology of tightly clustered cells. (A) Colony formation following treatment with 50ng/mL of EPO and either 10ng/mL (LD) or 100ng/mL (HD) of tumor necrosis factor-α (TNFα). (B) Colony formation following treatment with 50ng/mL of EPO and either LD or HD of interferon-γ (IFNγ). (C) Colony formation following treatment with 50ng/mL of EPO and either LD or HD of TNFα and IFNγ together. Data are represented as mean ± SEM in triplicate. Asterisks (*) denotes statistical significance ($p<0.05$) from EPO treatment alone, § denotes statistical significance ($p<0.05$) between treatment groups.

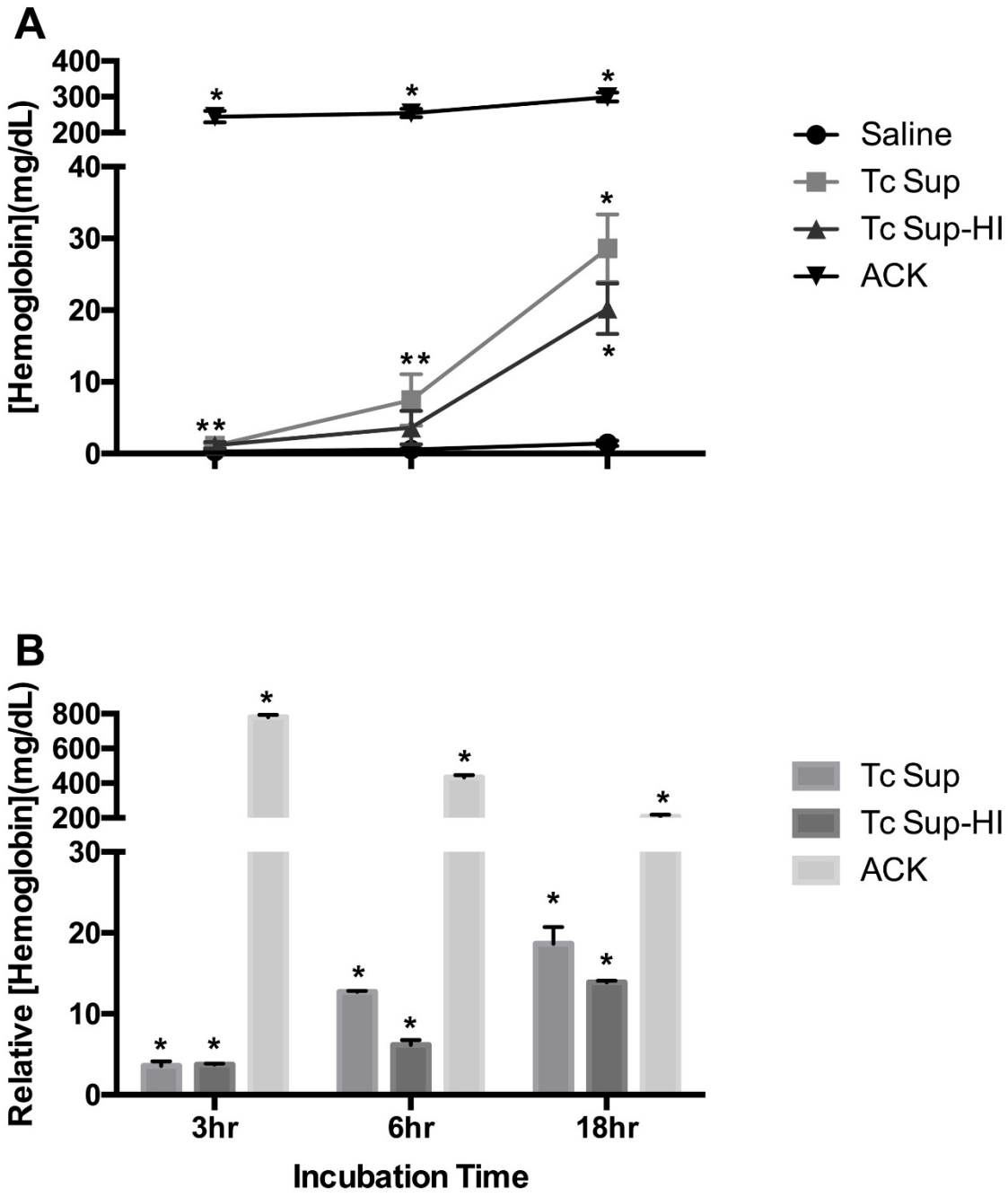

Figure 5.5 Hemoglobin release from goldfish erythrocytes *in vitro*.
Blood samples were mixed 1:1 with either non-heat inactivated (Tc Sup) or heat-inactivated (Tc Sup-HI) supernatants collected from *in vitro T. carassii* cultures. A subsample was isolated immediately after mixing as a baseline, and then at 3, 6, and 18 hours post-incubation at 20°C. Serum was collected, and hemoglobin values were determined by colorimetric change at 400nm using a Hemoglobin Assay Kit (Sigma-Aldrich). (A) Total hemoglobin release during incubation. (B) Relative lysis of treatment groups in relation to the saline control. Saline-0.9% NaCl; ACK-ACK Erythrocyte Lysis Buffer (Gibco). Asterisk (*) denotes statistical significance ($p<0.05$) from saline control.

CHAPTER 6

GENERAL DISCUSSION

6.0 Introduction

Infections of fish with *T. carassii* are generally non-lethal, and fish are able to control infections (7). However, the major pathophysiology of this parasitic infection is the onset of a long-lasting anemia, which can make the host more susceptible to secondary infections which can be significantly more lethal. My thesis research focuses on how fish erythropoietic system was altered in response to parasitic infection. My research findings include both an *in vitro* and *in vivo* analysis of the induction and maintenance of anemia in goldfish infected with *T. carassii*.

The main objective of my thesis was to determine whether the anemia observed during infection with *Trypanosoma carassii* is primarily mediated by the host, or by parasite derived factors. The specific aims were: (1) to examine the expression of key genes that regulate erythropoiesis during the course of infection with *T. carassii* in the goldfish; (2) to determine whether the changes in gene expression could be altered by administration of recombinant goldfish erythropoietin (rgEPO); and (3) to examine the possible effects of recombinant pro-inflammatory cytokine and parasite excretory/secretory products treatments on progenitor and terminal erythroid cells *in vitro*.

6.1 Overview and discussion of findings

Previous work with *T. carassii* revealed that decrease in red blood cells (RBCs) occurred in goldfish during the course of the infection (7). This decrease in the number of RBCs was shown to be sustained and paralleled the peak parasite load in the infected fish (198). Therefore, it was important to first assess whether there was an impairment in the expression of key genes involved in regulating erythropoiesis in fish during the different stages of infection with *T. carassii*. The mRNA levels genes encoding the RBC growth factor and its receptor, erythropoietin (EPO) and EPOR, were initially examined as well as those of GATA-1 and Lmo2 due to their critical roles in directing hematopoietic stem cell (HSC) differentiation towards the erythroid lineage (4-6). Additionally, the mRNA level of hypoxia inducible factor (HIFα) has previously been shown to regulate erythropoiesis during states of oxygen deprivation was also determined using qPCR (173). I also decided to determine the mRNA levels of interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) since they have previously been shown to be upregulated in response to *T. carassii* infection, and the studies using different trypanosome models have suggested that these cytokines contributed to pathophysiology of trypanosomiasis (230).

The results of gene expression analyses revealed decreases in the mRNA levels during early and acute phases of the infection for genes responsible for regulation of erythropoiesis. Under normal circumstances, expression of *epo* is upregulated in response to anemia, and is associated with restoration of erythrocyte numbers and recovery from acute anemia (129). To establish what the baseline response to anemia was in the infected goldfish, I decided to use phenylhydrazine (PHZ) to chemically induce hemolytic anemia and examine the same panel of genes. In this case, significant decreases in blood metrics resulted in massive increases in mRNA levels of the target genes almost immediately after

the onset of anemia – demonstrating that goldfish possess the ability to rapidly respond. However, *epo* expression in *T. carassii*-infected goldfish remained significantly decreased in the kidney and spleen during the induction of anemia which directly contrasted what was observed following treatment with PHZ. Although this result was unexpected from a physiological standpoint, a similar gene expression profile has been previously reported for other species of trypanosomes, suggesting that *Trypanosoma* spp. are capable of negatively influencing erythropoiesis in infected hosts (143,154).

When one examines the trends of gene expressions and changes in blood metrics during the infection it is evident that the parasite inhibited erythropoiesis, such that the host was unable to respond appropriately and restore the number of erythrocytes to homeostatic levels. This was further supported by the observation that during the resolution stage of infection, when the numbers of parasites decreased, the mRNA levels of EPO significantly increased in all tissues examined. This increase in *epo* expression paralleled a steady increase in both the packed cell volume (PCV) and total number of red blood cell (RBC). Previous studies have suggested that trypanosomes interfered with the transcription of the *epo* gene and were also capable of modifying and inactivating the EPO glycoprotein; both of which would have direct negative impact on the ability of the host to carry out proper erythropoiesis (143,231). Given that our lab previously generated and functionally characterized recombinant goldfish EPO (rgEPO), I decided to assess whether the observed anemia was due to inability of goldfish to generate sufficient amounts of EPO mRNA which could indicate insufficient production, and if so, whether the observed anemia in the fish could be reversed by administration of exogenous rgEPO.

rgEPO was administered during the early and acute stages of infection, when the blood metrics and gene expression decreased. Previous studies have demonstrated that EPO therapy during acute stages of *T. congolense* infection reduced both the morbidity and mortality associated with infection (181). In my study, the administration of exogenous rgEPO altered the kinetics of infection, causing a delay in the onset of peak parasitemia and anemia. Instead of preventing significant pathology, the administration of rgEPO caused a short-lived protective effect that reduced the severity of infection. These findings may be explained by the pleiotropic effects of EPO on the host. EPO also possesses anti-inflammatory properties, and has been shown to cause decreases in the production of NO, TNFα and IL-6 related cytokines (205). Treatment of rodents with recombinant EPO has been previously shown to facilitate these anti-inflammatory responses *in vivo* during infection. Rats with sepsis-induced liver injury receiving EPO injections had significantly reduced levels of TNFα, IFNγ, IL-1β, IL-6, IL-8 as well as several other inflammatory mediators (232). Therefore, administration of rgEPO during *T. carassii* infection may have promoted an anti-inflammatory state that was protective against severe anemia during the acute phase of infection. Knowing the potential negative effects of enhanced inflammation on host tissue/cells, and given that host-induced pathology was shown to be associated with many infections ; I decided to examine whether different pro-inflammatory mediators contributed to the etiology of *T.carassii*-induced anemia.

One of the key differences when gene expression profiles of *T. carassii* infection and PHZ treatment are compared was the expression of the proinflammatory genes IFNγ and TNFα. Previous studies have shown that trypanosome infection leads to the induction of a Th1 mediated immune response which includes a variety of proinflammatory cytokines

including TNFα, IFNγ, IL-1 and IL-6 (112). IFNγ and TNFα have both been shown to be important in defense against trypanosome infections. TNFα has been shown to be trypanolytic, while IFNγ was mainly involved in activation of macrophages to produce nitric oxide (NO) (236,237). Additionally, serum levels of these cytokines have been previously shown to be related to PCV changes in rabbits infected with *T. brucei* as well as in rats infected with *T. evansi* (191,238). One of the main contributing factors to this relationship may be the inhibitory effects that proinflammatory cytokines exert on erythropoietin production. TNFα has been shown to impact the production and secretion of EPO in erythroid cells (239). This activity was enhanced by the presence of IL-1, which has been previously shown to be also upregulated in response to *T. carassii* infection (111). IFNγ has also been shown to decrease the production of erythropoietin *in vitro*, and decrease EPOR on the surface of erythroid progenitor cells (240). These observations corroborate my findings of gene expression analysis, since *epor* mRNA levels were downregulated during the course of infection while the mRNA levels of IFNγ were elevated.

As a proinflammatory cytokine, TNFα has a positive effect on the DNA binding abilities of NFκB and GATA-2, which are highly conserved and important in mediating a plethora of immune pathways (241). Signaling through this NFκB promotes inhibition of erythropoiesis related gene expression, such as globins and EPO itself (242). GATA-1 and GATA-2 share binding sites, and the biding of GATA-2 early on in hematopoiesis prevents lineage commitment as GATA-2 functions primarily to maintain the pool of undifferentiated HSCs (243). However, due to this shared binding site, increased binding capacity of GATA-2 is capable of reducing EPO synthesis, and overexpression of GATA-2

redirects cells away from erythropoiesis and towards megakaryopoeisis (248,251). In addition to directly impacting EPO production and therefore erythroid differentiation, proinflammatory signaling can cause HSC lineage reprogramming. EPO and proinflammatory cytokines exert their effects through a conserved JAK/STAT pathway, which leads to the expression of different genes (172). Excessive stimulation of these inflammatory pathways can mop up secondary signaling molecules, leading to "signal hijacking" and a redirection of the HSC pool towards a myeloid lineage as opposed to a erythroid lineage (242). The cross-antagonistic relationship between PU.1 and GATA-1 has been previously established, with the production of PU.1 promoting the commitment to the myeloid lineage (245). Previous studies using *T. carassii* in our lab have shown that decreased expression of GATA-1 parallels the increases in the expression of PU.1 and GATA-2 in both the hematopoietic organs and sorted progenitor cells of infected fish, suggesting that these cells are being reprogrammed towards the myeloid lineage (253). The results of my *in vitro* assay support this theory, which showed the formation of diffuse myeloid colonies. Similar colonies have been observed in studies involving zebrafish cells that exhibited increased expression of GATA-2 and PU.1 (247).

The effects of proinflammatory cytokines also impacts cells on a more macro level. In addition to altering transcription and lineage commitment, they can also inhibit overall growth and differentiation of erythroid progenitor cells (248). I examined whether proinflammatory cytokine inhibitory effects were conserved in fish, by quantifying the erythroid colony formation in the presence of recombinant goldfish IFNγ (rgIFNγ) and TNFα (rgTNFα). I observed that these cytokines inhibited colony formation in methylcellulose cultures, and these effects were additive when recombinant cytokines were

added in combination. IFNγ has been shown to be the main suppressor of erythroid development, and functions primarily through decreased proliferation and the induction of apoptosis involving caspases 1, 3, and 8 (249). On the other hand, TNFα was shown to be primarily involved in decreasing the ability of cells to differentiate into the erythroid lineage, as well as mediating apoptosis through Fas/TRAIL ligands (225). Although EPO has been shown to exert protective, anti-apoptotic effects, the addition of rgEPO to cultures did not prevent decrease in in erythroid colonies caused by pro-inflammatory cytokines. It is possible that the concentration of rgEPO used in my experiments was too low to offset cytokine-mediated inhibitory effects. It is known that patients receiving erythropoiesis stimulating agents to combat anemia caused by chronic kidney disease, require higher than usual doses of stimulating agents (including EPO), in order to maintain proper hemoglobin levels (250). This was thought to be due to interference caused by excessive production of proinflammatory cytokines such as IL-1, TNFα and IFNγ. So, although cells were being treated with a dose of rgEPO previously shown to be optimal for colony formation, this may not have been enough to counteract the inhibitory effects of pro-inflammatory cytokines.

"Bottlenecking" at the progenitor stage is only a contributing factor to the anemia that is observed during *T. carassii* infection. Previous studies have shown that trypanosomes are capable of producing hemolysins causing direct lysis of RBCs in the vasculature (251). A hemolysin has been proposed for *T. carassii*, similar to what has been observed for *Trypanoplasma borrelia* (23). However, I believe that both mechanical damage and release of intracellular components, at concentrations significantly higher than would be biologically relevant during infection, are the major contributors to the observed

hemolysis in these *in vitro* studies. Regardless, the flagella of trypanosomes are capable of causing significant damage to the membranes of erythrocytes, and the release of free-fatty acids following parasite death or autolysis are potent agents that can contribute to membrane damage and eventual lysis of erythrocytes (252).

I also examined whether parasite-derived excretory/secretory molecules caused lysis of erythrocytes *in vitro.* The results showed that prolonged incubation of erythrocytes with parasite culture supernatant caused an increase in the levels of hemoglobin, suggesting that molecules present in parasite supernatant contributed to lysis of erythrocytes. Although further studies are be required to identify parasite-derived molecules that are responsible for the hemolysis. The observed hemolysis may be caused by the production of toxic metabolites by the parasites, or high concentrations of free iron in the serum which may have cytotoxic effects. These observed effects are likely exacerbated in an *in vitro* assay; however, in combination they could contribute to the lysis of erythrocytes in the vasculature and the observed decreases in circulating RBCs. This may be one of the mechanisms that trypanosomes utilize in order to liberate iron as a source of securing nutrients (116). Iron plays a key role in both normal hematopoiesis, specifically erythropoiesis, as well as during infection. Previous studies examining *T. carassii* infection parameters in goldfish have shown that there was a significant upregulation in acute phase proteins (APPs) that are involved in iron sequestration and storage (253). One such molecule, hepcidin, has been shown to be involved in both binding free iron and exerting trypanolytic effects (254). Macrophages serve as one of the main cells where iron is stored, and erythrophagocytosis is a mechanism by which these cells can acquire iron (155). Proinflammatory cytokines, specifically IFNγ, are known to upregulate

erythrophagocytosis and also increase the number of iron uptake transporters on macrophages (255). Heme serves as a key source of iron, and its release from erythrocytes can then be reused in order to fuel hematopoiesis. However, inflammation can alter the overall allotment of iron, leading to hoarding of iron within cells and overproduction of immune cells in lieu of erythrocytes (255). Increased RBC turnover in order to "steal" hemoglobin and increase the production of new immune cells via myelopoiesis, in conjunction with a decreased production of new erythrocytes, could function in tandem to significantly contribute to anemia during trypanosomiasis (256).

6.1.1 Model of *T. carassii*-mediated anemia

The findings of my research suggest that inflammation may be a significant contributor to *T. carassii*-mediated pathophysiology and specifically anemia. A model of this interaction between inflammation and pathophysiology is outlined in Figure 6.1. Increases in proinflammatory gene expression are induced in response to parasitic infection, and serve as the first line of defense in protection against invading pathogens (18). This cytokine profiles can be manipulated by the parasite in an attempt to avoid rapid immune clearance (25). This includes promoting a Th1 response, characterized by the production of cytokines such as IFNγ, TNFα, IL-1, and IL-6, which are generally involved in clearance of intracellular pathogens as opposed to extracellular pathogens like *T. carassii* (257). I observed increases in proinflammatory gene expression throughout the infection, which is supported by previous studies in goldfish infected with *T. carassii* (111).

Increased production of proinflammatory cytokines such as TNFα, IFNγ and IL-1, as demonstrated in my thesis research, can have inhibitory effects on the production of both

EPO its receptor EPOR (113,240), which would undoubtedly cause inadequate signaling through EPOR, resulting in an insufficient stimulus to promote commitment to the erythroid lineage. The gene expression results support this, since the *epor* expression remained consistently downregulated in the presence of IFNγ, but not TNFα. Additionally the presence of proinflammatory cytokines (i.e. TNFα) can affect the survival of existing erythroid colonies by inducing caspase mediated apoptosis (249,258). EPO has repeatedly been shown to exert strong anti-apoptotic effects in erythroid progenitor cells (259). Due to the fact that EPO is no longer being produced, it is unable to exert protective effects on the existing erythroid progenitor cells, and the presence of these proinflammatory cytokines leads to direct apoptosis of the erythroid progenitor population.

A concurrent shift in the intracellular signaling pathways of HSCs exacerbate this problem, whereby proinflammatory signaling reprograms hematopoiesis towards the myeloid lineage (260). Simultaneous degradation of the transcription factor GATA-1, coupled with increased expression and activity of PU.1 and GATA-2, induced by both TNFα and IFNγ, would redirect cells away from the erythroid lineage to the myeloid lineage (225,261,262). Our lab has previously reported on the cross-antagonistic activity of PU.1 on GATA-1, and ability to promote myelopoiesis in goldfish (245). Additionally, previous studies in goldfish challenged with *T. carassii* showed that infection caused significant decrease in GATA-1 expression in both the kidney and R1-gated progenitor cells during the early stages of infection, supporting the theory that these cells are being reprogrammed at the early stages of parasitic infection (246).

Taken together these data are supported by my *in vitro* colony assays which demonstrate a reduction in the number of erythroid colonies in the presence of rgIFNγ and

rgTNFα, as well as the formation of what appear to be myeloid colonies based on morphology previously described for zebrafish. However, the presence of exogenous EPO in colony assays did not offset cytokine induced apoptosis, which may simply be due to insufficient amounts of EPO relative to that of cytokines

Due to this multifaceted suppression of erythropoiesis at the progenitor level during infection, the host will have a decreased capacity to produce new terminal erythrocytes and restore blood metrics to homeostatic levels. *T. carassii* infections are long lasting, and antibody mediated clearance of the parasite does not generally kick-in until approximately day 28 post infection (24). Therefore, this Th1 inflammatory phase preceding the switch to a Th2 anti-inflammatory/antibody phase could be a significant contributor to the prolonged anemia observed during *T. carassii* infection.

The resolution stage of *T. carassii* infection in goldfish is characterized by the production of cytokines such as TGF-β and IL-10, which exhibit anti-inflammatory properties (111). Additionally, these cytokines in combinations with other cytokines, such as IL-3 and stem cell factor (SCF), have been shown to promote the production of terminal erythrocytes, even in the presence of inflammatory inputs (264,265). Thus, the onset of these anti-inflammatory mechanisms would facilitate a shift back to proper erythropoiesis and replenish erythrocytes as supported by the observed increase after day 28 of infection.

6.2 Future Directions

The role of inflammation in the context of parasite-induced anemia was not a significant focus until later in my research, but my findings in this area have been quite interesting and, I believe, warrant further investigation. The expression of pro- and some

anti-inflammatory cytokines has been previously explored in our lab (111). However, the period immediately after acute infection (days 28-56) remains relatively unexplored even though there was a clear transition away from the proinflammatory state at the onset of the recovery phase. Focusing on the change in expression levels of anti-inflammatory cytokines IL-10 and TGF-β and additionally IL-3, IL-4, IL-6 and SCF, which have all been shown to regulate both inflammation and erythropoiesis, during this critical time period would shed some light on when and how this transition occurs in the host. Of specific interest is IL-10, which has been shown to facilitate this switch and was shown to be essential for regulating tolerance during trypanosomiasis (266). Animals lacking IL-10 exhibit more severe immunopathological symptoms and decreased lifespan due to an inability to mediate the proinflammatory state induced by the Th1 response (267). Interestingly this cytokine has been shown to control parasite numbers and prolong survival in addition to preventing the onset of anemia (268,269). Recombinant IL-10 has previously been generated in our lab, and was shown to reduce the induction of proinflammatory responses *in vitro* (270). These studies also revealed that pre-treatment with IL-10 leads to decreased production of reactive oxygen intermediate (ROI) and IFNγ in activated immune cells. Administration of IL-10 prior to *T. carassii* infection and quantifying how this impacts parasitemia and pathophysiology would broaden our understanding of what role inflammation plays in the early stages of the infection in the goldfish.

Similarly, induction of a strong Th1 response has been proposed as a potential immune evasion mechanism by *T. carassii* and previous studies in our lab have suggested that the induction of a vigorous Th1 response in the early stages of infection was associated with an increased ability of the host to manage parasitic infection as demonstrated by

reductions in parasitemia (271). In my studies, a similar reduction in parasitemia was shown to occur with the administration of an anti-inflammatory molecule EPO. It would be interesting to examine whether EPO immunomodulation would cause a systemic shift in Th1 responses, or whether it solely functions to prolong survival of progenitor cells which was suggested by the short-lived effects of exogenous rgEPO administration.

T. carassii has previously been shown to produce molecules that are capable of both stimulating and inhibiting inflammatory responses (25). Glycosylphosphatidylinositol (GPI) moieties are produced by several species of trypanosomes, and have been shown to promote the onset of an M1 phenotype in exposed macrophages (272). However, timing has been shown to be important as pre-stimulation of macrophages by GPI before exposure to IFNγ has been shown to shift the macrophages to an alternatively activated M2 phenotype, characterized by a reduction in IL-12 synthesis and increased production of anti-inflammatory cytokines (273). Heat shock protein (hsp) 70 and glycoprotein (gp) 63 are produced by *T. carassii* and have been shown to have immunomodulatory effects, and previous work in our lab has shown that immunization with parasite excretory/secretory (ES) products leads to increased parasite clearance during the early stages of infection (27,147,148). Previous research has mainly focused on immunomodulatory effects and subsequent changes in the immune response to the parasites, whereas their role in host pathophysiology remains relatively unexplored. Therefore, examining the *in vivo* effects of hsp70 and gp63 administration, specifically focusing on their potential ability to induce M2 macrophage phenotype switch, and whether that influences hematological responses would be interesting to explore in the context of parasite-induced anemia. Linking this previous

research, with my current work would help explore the extent to which host responses play in promoting the onset and maintenance of anemia during *T. carassii* infection.

The M2 macrophages have also been reported to alter iron homeostasis, specifically by reducing iron-withholding and increasing bioavailability for proper hematopoiesis (274). Iron regulation has been previously demonstrated to be integral to the goldfish acute phase response (APR) to *T. carassii* infection (253). Exploring changes in the APR, specifically iron homeostasis, in response to anti-inflammatory priming, and ho this impacts host pathophysiology during infection with *T. carassii* would also provide valuable information about host contributions to anemia.

6.3 Conclusion

In conclusion, my research reports on the pathophysiology of *T. carassii* infection in goldfish and suggests that parasite-mediated inflammation plays a significant role in inhibiting erythropoiesis and promoting anemia in the host. Through both *in vitro* and *in vivo* analysis, I demonstrated that suppression in erythropoiesis coincides with enhanced inflammation and decrease in hematological parameters. I believe that my research findings have contributed to our understanding of the host-parasite relationship, and more specifically, the characterization of elements during the course of infection with *T. carassii* that contribute to host pathophysiology.

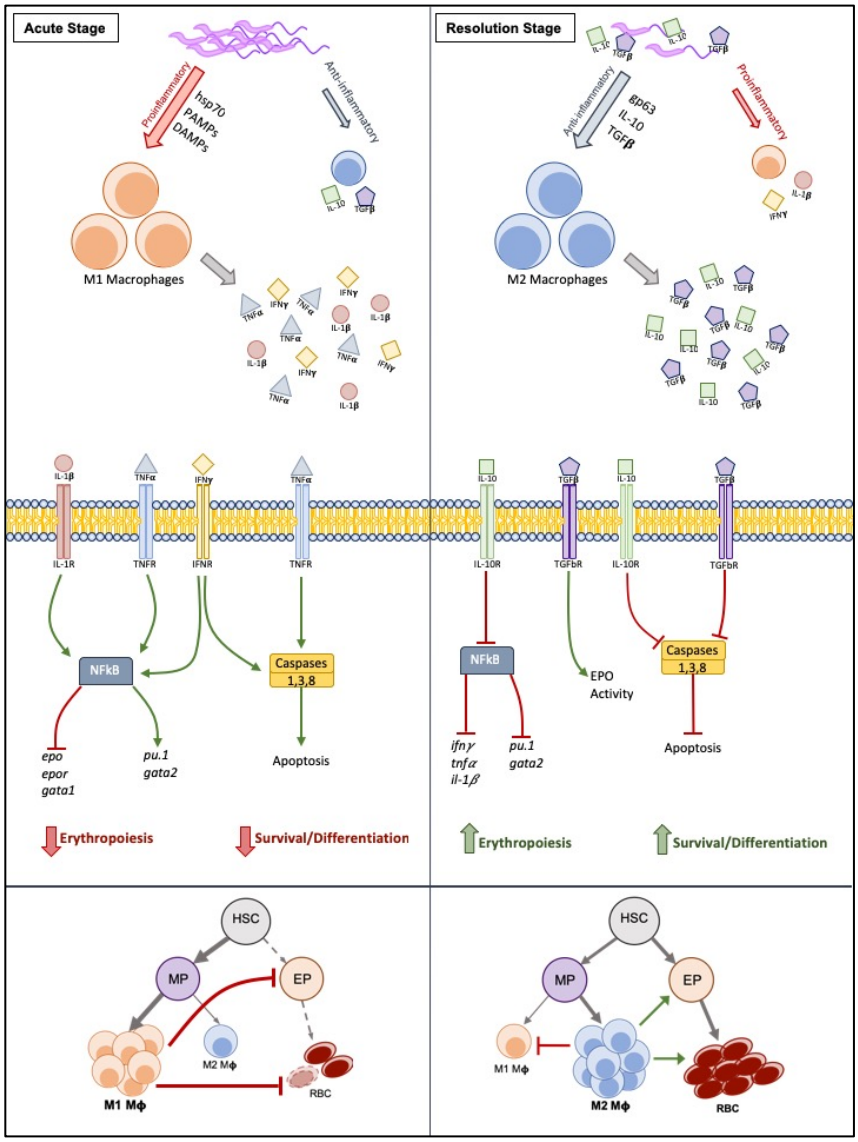

Figure 6.1 Model of inflammation mediated anemia during Trypanosoma carassii infection.

During the acute stage of *T. carassii* infection, increasing parasitemia causes production of proinflammatory mediators such as hsp70, PAMPs, and DAMPs which induce a Th1 response and cause M1 macrophage phenotype polarization. Subsequent production of proinflammatory cytokines promotes apoptosis and decreases expression of erythroid lineage genes in progenitor cells. During the resolution stage of infection, parasite numbers drop, and levels of anti-inflammatory mediators rise resulting in a shift to an M2 macrophage phenotype. Anti-inflammatory cytokines inhibit synthesis of proinflammatory cytokines, exert anti-apoptotic effects, and increase differentiation in progenitor cells. Hsp70 – Heat shock protein 70; PAMP – Pathogen Associated Molecular Pattern; DAMP – Danger Associated Molecular Pattern; gp63 – Glycoprotein 63; IFN – interferon; TNF – Tumor Necrosis Factor; IL – Interleukin; TGF – Transforming Growth Factor; HSC – Hematopoietic Stem Cell; MP – Myeloid Progenitor; EP – Erythroid Progenitor; RBC – Red Blood Cell

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