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FISH PARASITOLOGY - ORIGINAL PAPER

Trypanosoma carassii infection in goldfish (*Carassius auratus* L.) : changes in the expression of erythropoiesis and anemia regulatory genes

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Abstract

Trypanosoma carassii is a flagellated bloodstream parasite of cyprinid fish with pathogenesis manifesting primarily as anemia in experimentally infected fish. This anemia is characterized by decreases in the number of circulating red blood cells (RBCs) during peak parasitemia. We examined changes in the key blood metrics and expression of genes known to be important in the regulation of erythropoiesis. Increasing parasitemia was strongly correlated with an overall decrease in the total number of circulating RBCs. Gene expression of key erythropoiesis regulators (EPO, EPOR, GATA1, Lmo2, and HIFα) and proinflammatory cytokines (IFNγ and TNFα) were measured and their expressions differed from those in fish made anemic by injections of phenylhydrazine (PHZ). Significant upregulation of pro-erythropoietic genes was observed in PHZ-induced anemia, but not during peak parasitic infection. Previously, we reported on functional characterization of goldfish erythropoietin (rgEPO) and its ability to induce survival and differentiation of erythroid progenitor cells in vitro. Treatment of goldfish during the infection with rgEPO reduced the severity of anemia but failed to fully prevent the onset of the anemic state in infected fish. Proinflammatory cytokines have been implicated in the suppression of erythropoiesis during trypanosomiasis, specifically the cytokines TNFα, IFNγ, and IL-1β. Analysis of key proinflammatory cytokines revealed that mRNA levels of IFNγ and TNFα were upregulated in response to infection, but only TNFα increased in response to PHZ treatment. Synergistic activity of the proinflammatory cytokines may be required to sustain prolonged anemia. These findings provide insight into the relationship between *T. carassii* and host anemia and suggest that *T. carassii* may directly or indirectly suppress host erythropoiesis.

Keywords Erythropoietin · EPO · Trypanosome · Anemia · Gene expression · Packed cell volume · PCV · Proinflammatory

Introduction

Trypanosoma carassii is a parasitic hemoflagellate that naturally infects teleost fish including tench, goldfish, and carp (Woo and Ardelli 2014). *T. carassii* is vectored by blood-sucking leeches, such as *Hemiclepsis marginata*, which take up parasites while feeding on fish (Qadri 1962). Following replication in the crop, the parasites multiply and eventually migrate to the proboscis of the leech, and are transferred to the fish host during subsequent feeding (Hayes et al. 2014). *T. carassii* can reach up to 100% prevalence and can cause significant host morbidity and mortality in aquaculture setting (Agüero et al. 2002; Overath et al. 1998). Pathologies reported during experimental infections include anorexia and damage to hematopoietic organs such as the kidney and spleen (Dyková and Lom 1979; Islam and Woo 1991). We developed an in vitro cultivation system for *T. carassii* (= *T. danielewskyi*) (Bienek and Belosevic 1999). When injected intraperitoneally to goldfish, the cultured trypanosomes were shown to be infectious to goldfish in a dose-dependent manner (Bienek et al. 2002)

Anemia is one of the most prominent pathophysiological manifestations caused by *Trypanosoma* spp. infections, and has a complex etiology (Morrison et al. 2010). Anemia during *T. carassii* infection has been hypothesized to be caused by the

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release of a parasitic excretory/secretory hemolysin-like molecule(s) (Islam and Woo 1991), similar to what has been observed in other parasite species such as *Cryptobia salmositica* and *Trypanoplasma borreli* (Woo and Ardelli 2014). Other species of trypanosomes have also been shown to secrete molecules that possess hemolytic activity. *Trypanosoma cruzi* produces an acid-active hemolysin that functions at low pH forming pores in the cell membrane (Andrews 1990). While the predicted purpose of this molecule is to aid in the escape of the parasite from the phagolysosomes, it can also lyse erythrocytes when parasites and erythrocytes are co-incubated together during the exponential growth phase of the parasite in vitro (Andrews and Whitlow 1989). In addition, *T. congolense* and *T. brucei* can cause erythrocyte lysis, although this is mediated through the release of phospholipases following parasite autolysis rather than through the secretion of hemolytic molecules (Tizard et al. 1978). The resulting free fatty acids act like a detergent, and cause extensive damage to erythrocyte plasma membrane (Bezie 2014). Other proposed mechanisms include increased erythrophagocytosis caused by cleaved sialic acid residues on the surface of red blood cells (RBCs), as well as impairment of erythropoiesis (Habila et al. 2012; Stijlemans et al. 2015).

Erythropoiesis is a complex and highly regulated process that is essential for blood homeostasis through the continuous production of erythrocytes. A critical step in the regulation of erythropoiesis is the binding of erythropoietin (EPO) to its cognate receptor EPOR (Elliott et al. 2008). Human EPO was first cloned in 1985 (Chou et al. 2004), and since then homologous genes have been identified in a wide range of mammalian species as well as in several fish species. The first teleost EPO molecule was identified in pufferfish *Fugu rubripes* and was subsequently identified and cloned in zebrafish (*Danio rerio*) and goldfish (Chu et al. 2007; Katakura et al. 2013). Due to the critical role that EPO plays in erythropoiesis, it has been a target of interest in the prevention and treatment of anemia in a variety of models, including both teleost and mammalian systems including humans. For instance, in humans, recombinant EPO has been used to effectively treat patients suffering from anemia caused by renal disease and hormone-disrupting cancers (Marsh and Rascati 1999).

While a parasite-produced hemolysin has been previously suggested as a driver of host anemia, the mechanism through which *T. carassii* induces and sustains anemia remains relatively unexplored. Goldfish serve as an excellent model to study the pathology associated with *T. carassii* infection, and insights on the mechanisms through which pathogenesis occurs may be useful in design of control strategies for combating trypanosomiasis in aquaculture setting. To explore host-parasite dynamics leading to anemia in piscine trypanosomiasis, we examined the expression of erythropoiesis-related genes, along with several blood metrics, during *T. carassii* infection of goldfish (*Carassius auratus* L). The first objective of our study was to analyze changes in erythropoiesis regulator gene expression to determine whether *T. carassii* infection caused transcriptional changes in the host. In addition, we used the chemical phenylhydrazine (PHZ) to stimulate hemolytic anemia and measure the expression of the same panel of genes. Comparing parasitically induced anemia to chemically induced anemia provides a reference to determine whether these manifestations of anemia elicit a similar physiological response in the host, or whether different insults result in distinct responses. The second objective of our study was to assess the bioactivity of our recombinant goldfish EPO (rgEPO), and examine the effects of EPO administration on the course of *T. carassii* infection. rgEPO has been previously cloned and characterized in our laboratory and has been shown to increase the proliferation, survival, and differentiation of erythroid progenitor cells (Katakura et al. 2013). The impacts of rgEPO administration during the acute stages of infection will help begin to explain whether the observed anemia can be attributed to the host, the pathogen, or a mixture of the two. We originally hypothesized that anemia was due solely to hemolysis mediated by *T. carassii*; however, the results of our study suggest that alterations in host physiology may play a more significant role in mediating anemia specifically in the context of host inflammation.

Materials and methods

Fish

Goldfish (*Carassius auratus L.*) (3–6 cm in length) were purchased from Aquatic Imports (Calgary, Alberta, Canada) and maintained in the Aquatics Facility in the Biological Sciences Building at the University of Alberta. Fish were kept in non-flow-through water system at 18 °C, on a simulated natural photoperiod (14-h light, 10-h dark). Fish were acclimated for 1 week before used in experiments. Prior to handling or manipulation, fish were sedated by immersion in water containing 50 mg/L of TMS (MS-222). The care of experimental animals followed guidelines of the Canadian Council of Animal Care (CCAC-Canada) and University of Alberta Animal Ethics protocol AUP069.

Parasites

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 (Bienek et al. 2002). Parasite cultures were incubated at 20 °C and passed every 6–7 days.

Infection of fish with *T. carassii*

Fish were anesthetized with TMS and then injected intraperitoneally using a 25-GA needle and tuberculin syringe with either low dose (LD) of 6.25×10^6 parasites or high dose (HD) of 1×10^7 parasites in 100 μL of TDL-15 medium. Control fish were injected with 100 μL of TDL-15 medium alone. Blood was then collected on days 4, 7, 14, 28, 42, and 56 post infection from the caudal vein using a heparinized 25-GA needle and tuberculin syringe to examine intensity of infection. Following collection, blood was transferred to a 0.6-mL microtube for further sampling. Parasitemia was measured by spinning a 70 μL sample of blood in a microhematocrit capillary tube at 10,000 rpm for 5 min. 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 was enumerated using a Neubauer hemocytometer, log transformed, and then presented as mean ± SEM parasites per milliliter 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.

RNA extraction, cDNA synthesis, and quantitative PCR

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 on days 7, 28, and 56 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-h 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 micrograms 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. In addition, PCR products for each gene were sequenced, to ascertain that correct genes were amplified. Primers were chosen if the R2 values were greater than 0.970, and efficiency was between 90 and 110%. qPCR cycling parameters were as follows: 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 (Hodgkinson et al. 2017; Oladiran et al. 2011) and others (Ghosh and Halpern 2016). All samples were run in triplicate and presented as the mean ± SEM of 4 fish for medium control and high-dose groups.

Genes were selected due to their involvement in the erythropoietic pathway, and are listed in Supplementary Table 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 (Ransom et al. 1996). GATA1 is essential for proper erythroid development, and loss of GATA1 in zebrafish represses erythropoiesis and pushes cells towards development into the myeloid lineage (Galloway et al. 2005). Lmo2 acts in a similar fashion to GATA1, and is also important for erythroid differentiation (Brandt and Koury 2009). Hypoxia inducible factor (HIFα) is a transcriptional regulator that is activated in in both mammals and fish in a state of oxygen deprivation, and leads to increased EPO production (Chu et al. 2008). 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 (Jelkmann 1998; Oladiran and Belosevic 2012).

Phenylhydrazine

Goldfish were injected with 12.5 μg/g of phenylhydrazine (PHZ), as has been previously described (Simonot and Farrell 2007). In brief, 100 μL of PHZ dissolved in 0.9% sterile saline solution was injected intraperitoneally. Blood and tissue samples were collected at 48-h post-injection to determine PCV and RBC number, and assess the mRNA levels using quantitative PCR.

Production and purification of recombinant goldfish erythropoietin

Recombinant goldfish EPO (rgEPO) has been previously produced and purified as described (Katakura et al. 2013). 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 1× 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 the 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) (Supplementary Fig. 1, Supplementary Table 2).

Administration of rgEPO

Goldfish were weighed, and average weight was used to determine injection volumes. Fish receiving EPO treatments were injected with 100 ng/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 1×10^7 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 1×10^7 parasites displayed a more severe anemia in preliminary studies, and was therefore chosen to test physiological effects of rgEPO.

Determination of blood metrics

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 0.6-mL microtube so that samples could be taken for the following assays. Packed cell volume was determined by transferring a 70 μL blood sample to a heparinized microhematocrit capillary tube (Fisher). Capillary tubes were then centrifuged at 10,000 rpm for 5 min, 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 (Houston and Murad 1992). Briefly, 10 μL 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 2 μL sample of blood diluted 1:100 in nuclease-free water (Ambion) was mixed with reagent and incubated for 5 min. Absorbance was then measured at 400 nm using a SpectraMax M_2 microplate spectrophotometer (Molecular Devices). Mean erythrocyte volume (MEV) was derived from the hemoglobin values using the formula (Murad and Houston 1992): $\mathrm{MCV} = \frac{\mathrm{Hematocrit}(\%) \times 10}{\mathrm{RBC}\ \left(\times 10^{12}/\mathrm{L}\right)}$.

Statistical analysis

Statistical analysis for blood metrics was performed by 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. qPCR results were analyzed by unpaired multiple t test, with a probability level of $P < 0.05$ considered significant. Pearson's r correlation analyses were used to establish r and R^2 values, with a probability level of $P < 0.001$ considered significant.

Results

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 (Fig. 1a, b; Suppl. Fig. S2). EPO and its receptor EPOR, and GATA1 and HIFα, were significantly down regulated 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 (Fig. 1c; Suppl. Fig. S3). 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 (Fig. 1b).

In the liver, mRNA levels remained relatively static for erythropoiesis-associated genes at 7 and 28 dpi (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 3a). EPO mRNA levels remained decreased at 28 dpi, whereas the expression of Lmo2 increased (Fig. 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) (Fig. 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 (Fig. 3b).

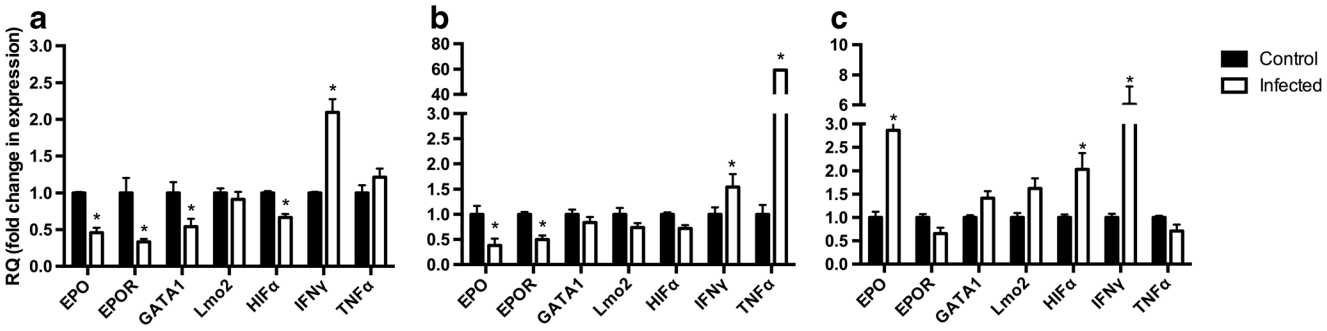

Fig. 1 Kidney mRNA levels of genes encoding erythropoiesis regulators in *T. carassii*-infected and -non-infected goldfish **a** 7 dpi, **b** 28 dpi, and **c** 56 dpi. Expression was relative to endogenous control gene, EF-1α, and normalized against those observed in non-infected control fish for each 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)

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 (Latunde-Dada et al. 2004; Simonot and Farrell 2007). The kidney showed increased levels of EPO and its receptor EPOR, along with the transcription factor GATA1 (Fig. 4a). Similar increases were observed in the spleen, with the addition of HIFα (Fig. 4c). The liver displayed the greatest changes in EPO expression with a 39-fold increase in mRNA levels at 48-h post PHZ injection (Fig. 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 the kidney, liver, and spleen following PHZ injection with the most significant increase occurring in the spleen (Fig. 4c).

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 6.25×10^6 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 (Fig. 5). Fish injected with 1×10^7 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 (Fig. 5). Recovery was similar to that observed for low dose, with blood metrics returning back to above baseline levels 56 dpi. The 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 (Fig. 6). Parasitemia was negatively correlation to both PCV and RBC counts, with a R^2 value of 0.5 and 0.43 respectively (Fig. 7a, b).

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

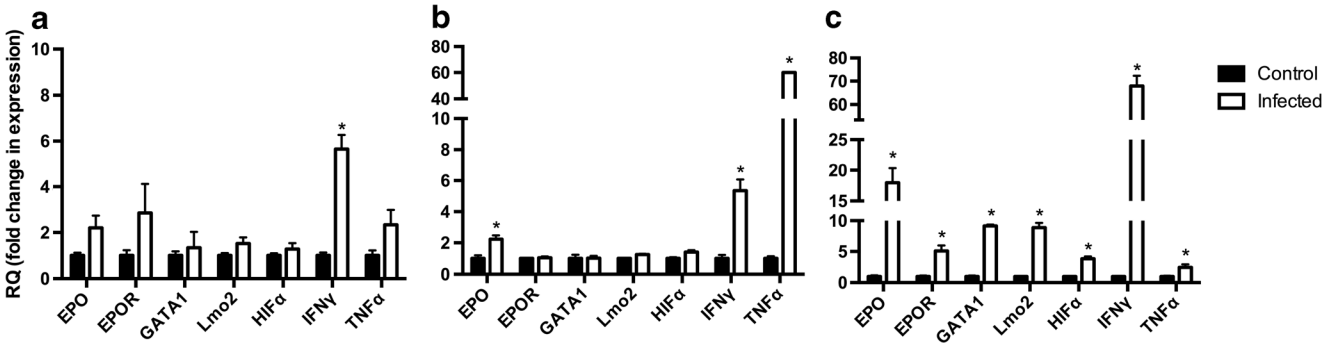

Fig. 2 Liver mRNA levels of genes encoding erythropoiesis regulators in *T. carassii*-infected and -non-infected goldfish **a** 7 dpi, **b** 28 dpi, and **c** 56 dpi. Expression was relative to endogenous control gene, EF-1α, and normalized against those observed in non-infected control fish for each 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)

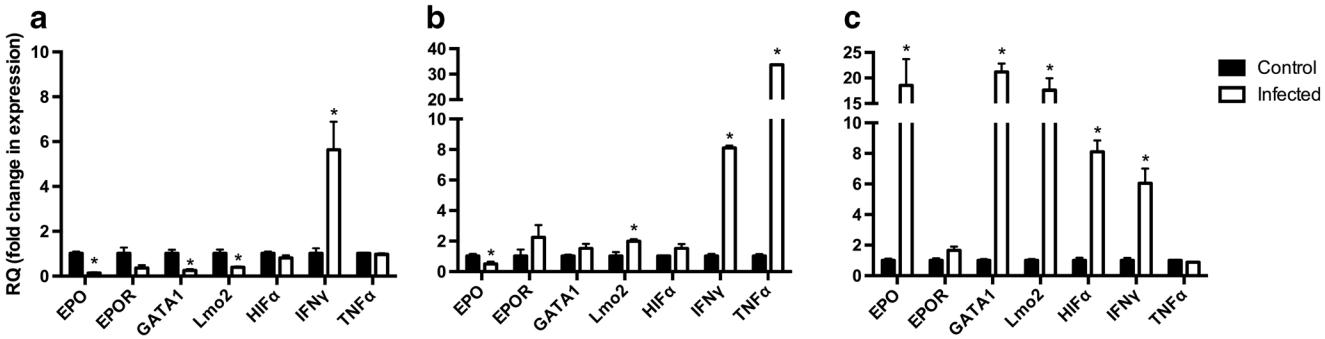

Fig. 3 Spleen mRNA levels of genes encoding erythropoiesis regulators in *T. carassii*-infected and -non-infected goldfish **a** 7 dpi, **b** 28 dpi, and **c** 56 dpi. Expression was relative to endogenous control gene, EF-1α, and normalized against those observed in non-infected control fish for each 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)

(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 (Fig. 8a). 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 (Fig. 8b).

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 (Fig. 8a, b). Following the 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 ($3.41 \times 10^7 \pm 1.6$ parasites/mL in EPO treated vs. $2.77 \times 10^8 \pm 0.9$ parasites/mL in untreated) recorded at 28 dpi (Fig. 9). The number of parasites subsequently spiked to a max of $3.45 \times 10^8 \pm 2.6$ parasites/mL at day 42 before beginning to decline, similar to what was observed in untreated fish.

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 (Fig. 10a). 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

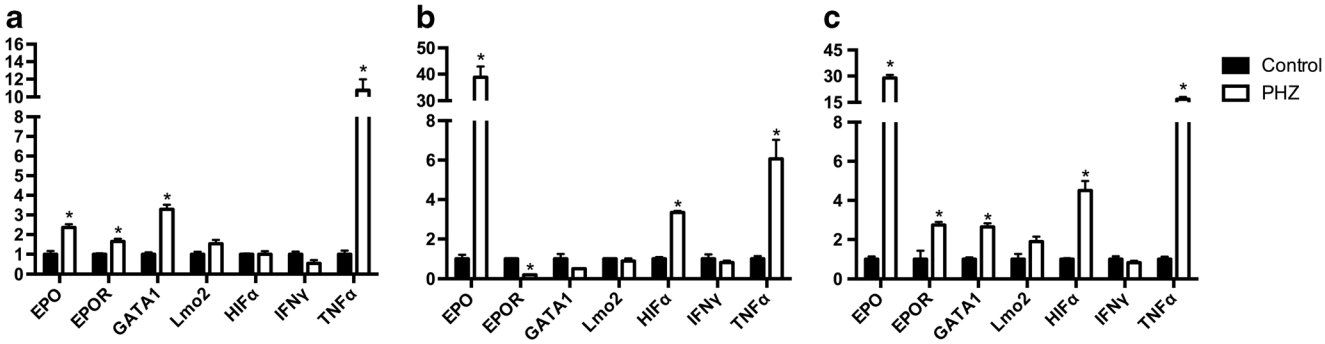

Fig. 4 Kidney (**a**), liver (**b**), and spleen (**c**) mRNA levels of genes encoding erythropoiesis regulators 48-h post-injection of PHZ to goldfish. Expression was relative to endogenous control gene, EF-1α, and normalized against those observed in non-infected control fish for each gene. Data are 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)

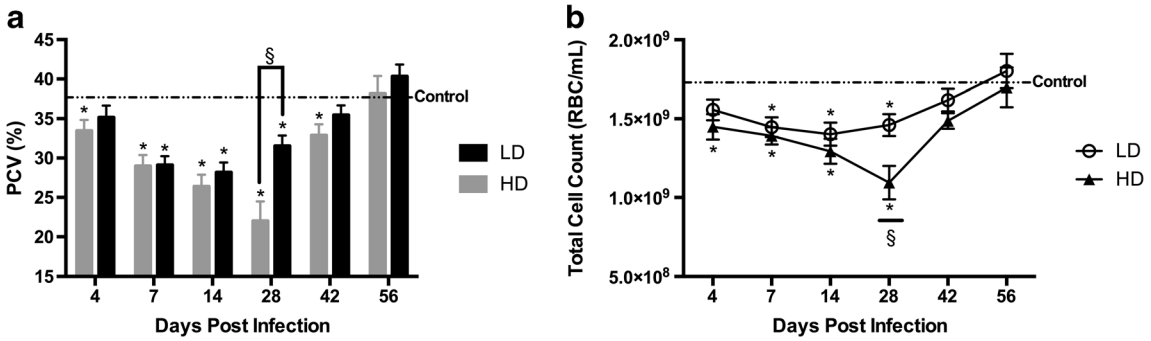

Fig. 5 Decrease in blood metrics are dependent on parasite dose. Treatment groups were inoculated with either 6.25×10^6 (LD) or 1×10^7 (HD) of *T. carassii*. Control groups were inoculated with TDL-15 medium. Control groups across all days were averaged and presented as a dotted line. Asterisk (*) denotes statistical significance ($P < 0.05$) from medium control. § denotes statistical significance ($P < 0.05$) between treatment groups ($n = 8$–12 fish per group)

in hemoglobin concentration was observed at 42 dpi, followed by a return to baseline levels at 56 dpi.

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 (Houston and Murad 1995). The MEV for *T. carassii*-infected fish remained relatively constant around 212 fL throughout the infection, with a slight decrease observed at 56 dpi (Fig. 10b). 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.

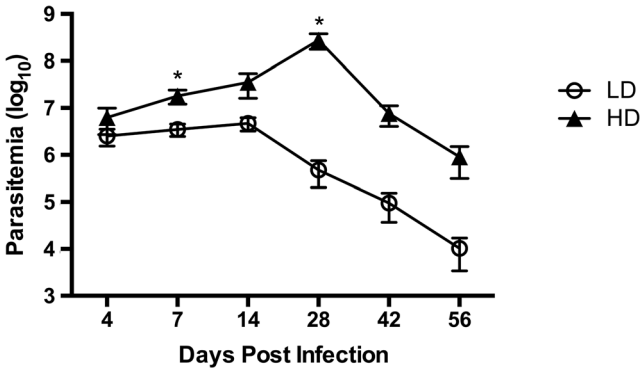

Fig. 6 Infection intensity is dependent on initial dose of *T. carassii*. Goldfish were inoculated with either 6.25×10^6 (LD) or 1×10^7 (HD) parasites. Microhematocrit capillary tubes were filed at the RBC/serum interface, and the serum including the parasites was diluted in PBS. Parasites were then 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 treatment groups ($n = 8$–12 fish per group)

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. The aim of our study was to characterize the progression of this anemic state during *T. carassii* infection by examining the expression of key genes involved in erythropoiesis alongside blood metrics. Our findings support the hypothesis that parasite presence exerted a suppressive effect on erythropoiesis, either directly or indirectly, as evidenced by changes in the expression of genes that are essential in erythropoiesis. The prolonged anemia and down-regulated gene expression observed during infection directly contrasted the short-lived anemia observed following PHZ treatment. Additionally, our results show that treatment of fish with rgEPO did not fully prevent the onset of anemia. This study suggests that anemia is not exclusively mediated by the parasite as previously thought, but rather the interactions between the changes in host physiology and the parasite, which leads to significant changes in host homeostasis.

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 (Suzuki et al. 2006). 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 (Noyes et al. 2009). Sudden decrease 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 (Jelkmann 2004; Nishimura et al. 2011). Although there was a significant decrease in the number of circulating blood cells following infection with *T. carassii*, EPO expression was not upregulated

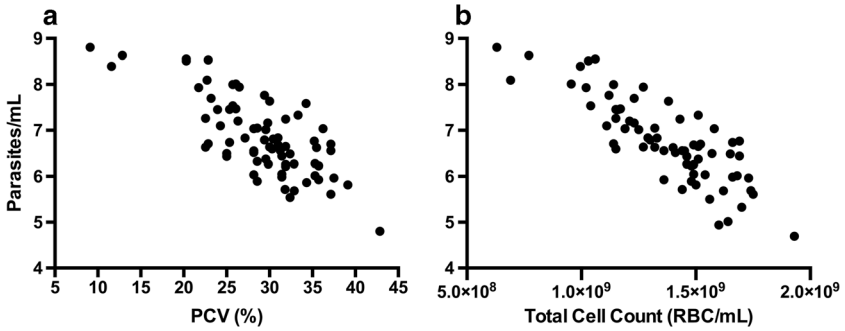

Fig. 7 Parasitemia is related to both PCV values (**a**) and RBC numbers (**b**). Parasitemia and blood metric data from individual fish at each day post infection were matched with one another and correlational analysis was performed to determine correlational coefficient (r) and coefficient of determination (R^2). Values were determined to be statistically significant ($P < 0.0001$). **a** $r = -0.71$, $R^2 = 0.50$. **b** $r = -0.65$, $R^2 = 0.43$

during the acute stages of infection. The resolution phase of infection however was characterized by a simultaneous increase in EPO expression and circulating blood cells, suggesting that the restraint on erythropoiesis is limited to the acute stages of infection and anemia, which directly contrasted what was seen 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 (Berger 2007). 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 (Houston and Murad 1995). 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 (Krasnov et al. 2013; Nogawa-Kosaka et al. 2010). In the fish, EPO binds its receptor erythropoietin receptor (EPOR) expressed on erythroid cells in the head kidney (the hematopoietic organ of fish), resulting in proliferation, differentiation, and maturation of erythroid progenitor cells (Katakura et al. 2013). 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 (Eckardt and Kurtz 2005). 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 (Nishimura et al. 2011). 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 intracellular and extracellular protozoans, and has been previously shown to increase during trypanosomiasis (Oladiran

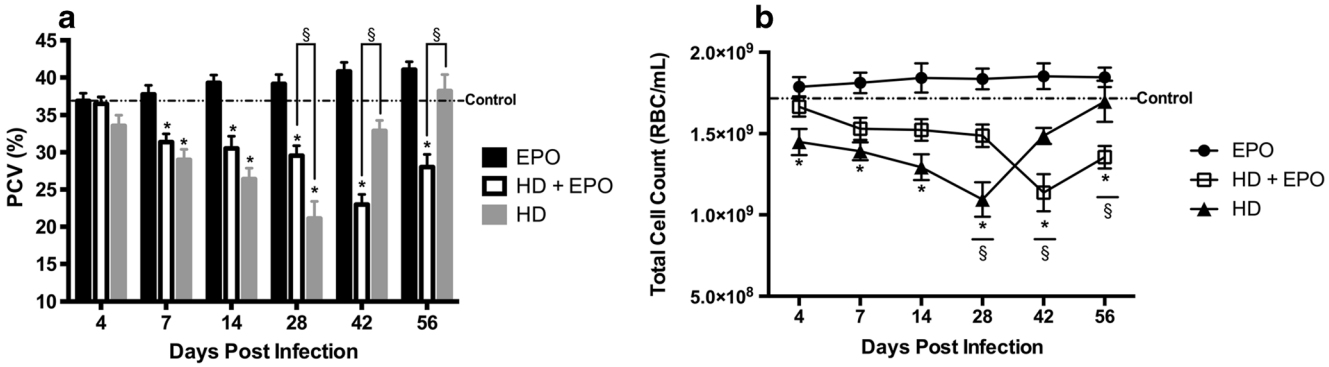

Fig. 8 Recombinant goldfish erythropoietin (rgEPO) injections delay the onset of anemia in fish infected with *T. carassii*. Treatment fish were injected with either 1×10^7 parasites at day 0 or 100 ng/g of rgEPO at days 0, 4, 10, and 24 (HD and EPO groups), or were injected with both parasites and EPO together (HD + EPO group). Control groups were sham injected 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 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)

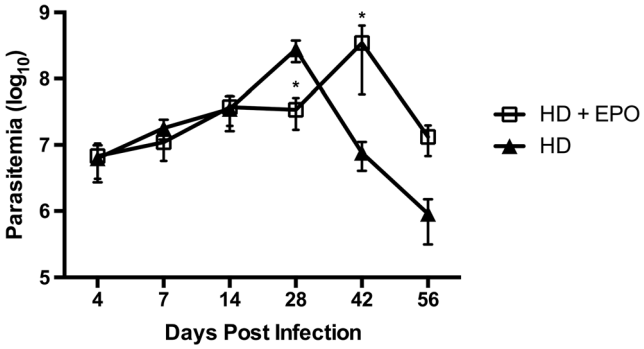

Fig. 9 Recombinant goldfish erythropoietin (rgEPO) injections alter the kinetics of parasitemia. Fish were injected intraperitoneally with 1×10^7 parasites at day 0. Microhematocrit capillary tubes were filed at the RBC/serum interface, and the serum including the parasites was diluted in PBS. Parasites 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 treatment groups ($n = 8$–12 fish per group)

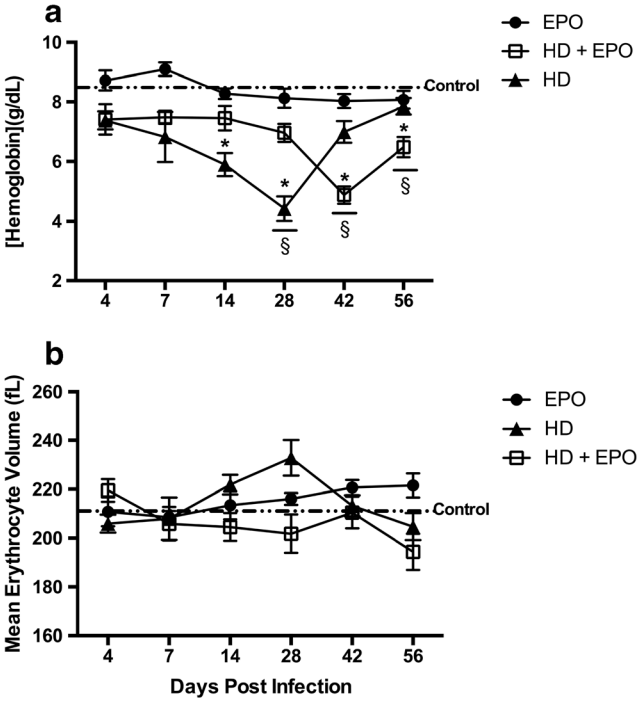

Fig. 10 Infection of goldfish with *T. carassii* causes a decrease in hemoglobin with no significant changes in mean erythrocyte volume (MEV). **a** Hemoglobin values were determined by colorimetric change at 400 nm using a Hemoglobin Assay Kit (Sigma-Aldrich). **b** MEV was determined using a standard formula described in the "Production and purification of recombinant goldfish erythropoietin" section of Materials and 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)

et al. 2011). The main function of IFNγ during infection in goldfish is to activate macrophages stimulate the production of nitric oxide (NO) (Oladiran and Belosevic 2012). However, IFNγ has also been shown to inhibit erythroid cell development in vitro by promoting apoptosis of hematopoietic progenitor cells (Cooper et al. 2003). Previous studies have demonstrated that trypanotolerant cattle resistant to *T. congolense* infection regulate erythropoiesis better than trypanosusceptible cattle (Boran) due to higher IFNγ mRNA levels that caused downregulation of the erythropoietic pathway (Suliman et al. 1999). 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 (Magez et al. 1999). Our results show that there is a significant increase in TNFα mRNA levels that coincide with peak parasitemia, followed by a decrease in both TNFα mRNA levels and parasite numbers. Additionally, serum TNFα levels have been previously demonstrated to be related to PCV changes in rats infected with *T. evansi* (Paim et al. 2011). 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 (Jelkmann 1998). 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 (Oladiran et al. 2011). 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.

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 (Chamond et al. 2010). 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 (Fig. 7a, b). 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 (Nogawa-Kosaka et al. 2011). 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 (Chang et al. 2004). 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 hematocrit observed in non-treated infected mice (Suzuki et al. 2006). 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. These findings may be explained by the pleiotropic effects of EPO on the host. In addition to stimulating erythropoiesis, EPO has demonstrated anti-inflammatory properties, and leads to decreases in the production of NO-, TNFα-, and IL-6 related cytokines (Nairz et al. 2012). Recombinant EPO treatment 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, and IL-8 as well as several other inflammatory mediators (Xiao et al. 2017). Therefore, administration of rgEPO during *T. carassii* infection may have induced an anti-inflammatory state that would help explain the less severe anemia observed during the acute phase of the infection.

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 (Groff and Zinkl 1999). 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 hematocrit, which remained decreased for an extended period of time (Khan et al. 1980). Additionally, *Hypostoma* spp. infected with *Trypanosoma* spp. demonstrated decreases in hb that were correlated with parasite intensity (Corrêa et al. 2016). These findings have also been observed in mammalian systems, specifically in water buffalo infected with *T. evansi* (Hilali et al. 2006). 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 (Murad 1990; Witeska 2013).

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 (Murad 1990). 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 (Nairz et al. 2012). 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 (Langousis and Hill 2014).

In summary, this study demonstrated that *T. carassii* infection in goldfish causes suppression of erythropoiesis regulator gene expression along with an increased expression of proinflammatory genes, similar to what has been reported for trypanosomes of homeothermic hosts. This suggests that the mechanisms that cause anemia during trypanosomiasis are evolutionarily conserved. Our findings indicate that proinflammatory cytokines are important in contributing to maintenance of the anemic state. The precise mechanisms as to how *T. carassii* induces anemia in poikilothermic hosts has yet to be elucidated, and to this end we are currently isolating and identifying parasite factors that may be directly involved in propagating the anemic state during piscine trypanosomiasis.

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