FLAVOHEMOGLOBIN EXPRESSION IN *GIARIDA INTESTINALIS* EXPOSED TO NITROSATIVE STRESS

A Thesis Submitted to the Committee of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Arts and Science

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ABSTRACT

Flavohemoglobin expression in *Giardia intestinalis* exposed to nitrosative stress

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The parasitic protist *Giardia intestinalis* lacks most heme proteins yet encodes a flavohemoglobin (gFlHb) that converts nitric oxide to nitrate and likely protects the cell from nitrosative stress. In this work an antibody raised against gFlHb was used to examine both changes in gFlHb expression levels and intracellular localization in Giardia in response to nitrosative stress. Giardia trophozoites exposed to stressors which either directly release nitric oxide (diethyltriamine NONOate, 1 mM) or are sources of other reactive nitrogen intermediates (sodium nitrite 20 mM or S-nitrosoglutathione, 1 or 5 mM) exhibited a 2 to 9-fold increase of gFlHb after 24 hours. Increased expression levels of gFlHb were detectable by 8 hours in S-nitrosoglutathione and diethyltriamine-NONOate-treated trophozoites, and by 12 hours after sodium nitrite exposure; these differences were likely due to differences in the rates of release of RNS from these compounds. In addition to a band of the expected size for gFlHb (52 kDa), western blots detected a second, higher molecular weight band (72 kDa) with comparable or higher intensity upon treatment with these RNS donors, which is consistent with sumoylation of gFlHb. Immunofluorescence microscopy of Giardia trophozoites detected gFlHb diffused throughout the cytoplasm and more punctuated staining along the cell membrane and between the nuclei. The punctuated staining may be due to the association of gFlHb with either peripheral vacuoles or basal bodies.

Keywords Nitrosative stress, Flavohemoglobin, *Giardia intestinalis*

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COMMON ABBREVIATIONS

ATP: Adenosine triphosphate

DETA-NONOate: diethyltriamine NONOate

flavoHb: flavohemoglobin

eNOS: epithelial nitric oxide synthase

FAD: flavin adenine dinucleotide

Fe^{3+}: ferric iron

Fe^{2+}: ferrous iron

FNR: ferredoxin: NADPH reductase

GAR-HRP: goat anti-rabbit horse radish peroxidase

gFlHb: Giardia flavohemoglobin

GSH: glutathione

GSNO: S-Nitrosoglutathione

HNO_2: nitrous acid

H_2O_2: hydrogen peroxide

iNOS: inducible nitric oxide synthase

ISC: iron sulfur clusterL-Cys: S-nitroso-L-cysteine

NADH: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

$NaNO_2$: Sodium nitrite

NO or $NO^{\bullet}$: nitric oxide

NO^-: nitroxide

NO^+: nitrosonium ion

NO_2: nitrogen dioxide

NO_2^-: nitrite

NO_3^-: nitrate

N_2O_3: dinitrogen trioxide

NOS: nitric oxide synthase

nNOS: neural nitric oxide synthase

O_2: oxygen

$O_2^{\bullet-}$: superoxide

$ONOO^-$: peroxynitrite

PV: peripheral vacuole

ROS: reactive oxygen species

RNS: reactive nitrogen species

SUMO: small ubiquitin-like modifier

ZIF: zinc finger protein

INTRODUCTION

1.1 Overview

Within the upper small intestine, the protozoan parasite *Giardia intestinalis* can experience nitrosative stress from various sources of reactive nitrogen species (RNS). Such sources include host nitric oxide synthases, stomach dietary contents, and the metabolism of other intestinal microflora. To counter the effects of nitrosative stress Giardia has a heme-containing flavohemoglobin. This protein has been shown in *Escherichia coli* to efficiently oxidize nitric oxide (NO) to nitrate (NO_3^-), and it is probable that Giardia flavohemoglobin (gFlHb) has a similar role.

1.2 *Giardia intestinalis*

Giardia intestinalis is an early divergent protozoan parasite that lacks many of the organelles associated with eukaryotes such as nucleoli, peroxisomes and mitochondria (Adam 1991). The organism has a simple two-stage life cycle that alternates between the infectious dormant cyst, and the metabolically active trophozoite. Ingestion of contaminated food or water containing cysts by a host causes the disease known as giardiasis. The acidic environment of the host's stomach causes the cyst to excyst into flagellated trophozoites. These trophozoites adhere, by their ventral disk, to the mucosa of the small intestine, particularly the duodenum and jejunum. In this environment there are fewer competing microorganisms compared to the large intestine, and because of the high nutrient content of the small intestine Giardia is able to derive its nutrients from this location in the digestive tract.

As a result, Giardia lacks many metabolic pathways found in most eukaryotes. Since Giardia lacks mitochondria, ATP is not generated by oxidative phosphorylation but by substrate level phosphorylation and fermentation pathways (Jarrol, Manning et al. 1989). Many biosynthetic pathways are also absent in Giardia, including that for heme biosynthesis. In eukaryotes most of the steps for this pathway occur in the mitochondria, which is also the site of the highest demand for heme. As Giardia lacks mitochondria, and it tests negative for common heme proteins such as catalase, heme proteins were not expected to be found in the Giardia genome. However, Giardia encodes at least five such proteins; four are members of the cytochrome b_5 family (electron transfer proteins) and the other is a flavohemoglobin protein (Morrison, McArthur et al. 2007, Rafferty and Dayer 2015). Flavohemoglobins are enzymes that detoxify nitric oxide, and is the sole heme-containing enzyme discovered in Giardia to date.

1.3 Nitric Oxide and Nitrosative Stress

Nitric Oxide (NO) is a gaseous free radical which has many roles in mammals including regulation of vascular tone, neurotransmission and immune response (Ridnour, Thomas et al. 2004, Stojanovic, Ljubisavljevic et al. 2012). Nitric oxide is synthesized in mammals from L-arginine by nitric oxide synthases (NOS) (Kleinert, Pautz et al. 2004). There are three types of NOS present in mammals: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). NOS is primarily expressed in neurons for retrograde signalling across neuron synapses and eNOS is expressed in endothelial cells for regulation of vascular tone. Both nNOS and eNOS are low output (picomolar) NO producers and are calcium/calmodulin dependent enzymes producing NO in a pulsatile nature. Inducible NOS is associated mainly with macrophages, although it can be

expressed in many cell types, including hepatocytes, smooth muscle tissue, bone marrow cells, keratinocytes and cardiac myocytes (Forrester and Foster 2012). Stimulation of macrophages by different infectious agents, which can be mimicked by a combination of bacterial lipopolysaccharide and interferon-γ, leads to strong expression of iNOS within several hours and to the steady production of micromolar quantities of NO. This is used as part of the immune response against certain pathogens and it also has roles in wound and tissue healing. Unlike nNOS and eNOS, inducible NOS activity is not regulated by calmodulin.

Within the small intestine Giardia trophozoites could encounter NO from a variety of sources (Nussler and Billiar 1993, Kim, Kim et al. 2002, Kleinert, Pautz et al. 2004). The major source of NO is likely generated by iNOS in macrophages and smooth muscle tissue. However, other microorganisms found in the gut can also produce NO during anaerobic respiration in which nitrogen oxides undergo reduction to NO by nitrite or nitrate reductases (Forrester and Foster 2012).

Dietary nitrate or nitrite can also be sources of NO. Nitrate does not directly produce NO, but it can be reduced to nitrite by bacterial nitrate reductases in the saliva. Within the acidic conditions of the stomach nitrite can be protonated to form nitrous acid, which decomposes into NO and nitrogen dioxide (NO_2). As a result, the amount of NO in the human stomach is relatively high (between 10-100 ppm). Nitrogen oxides can also be found in the gut lumen on the mucosal surfaces by selective accumulation of endogenous and dietary derived nitrogen species (Lundberg and Weitzberg 2013, Pereira, Ferreira et al. 2013).

Nitric oxide is a small and uncharged free radical that can easily pass through cell membranes (Kim, Kim et al. 2002). While NO has the potential to damage several types of biological molecules due to its free radical nature it is safe at the low (picomolar) concentrations used for cell signalling roles, which are mediated by reversible binding to the heme cofactor of soluble guanylate cyclases; such binding stabilizes the unpaired electron on NO. At micromolar concentrations NO can react with transition metals, other radicals and reactive oxygen species (ROS). In particular, reactions with ROS such as molecular oxygen (O_2), superoxide ($O_2^{\bullet -}$) and peroxide (H_2O_2) can produce other agents such as a nitrosonium ion (NO^+), nitrite (NO_2^-), dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$). As the fates of NO within the cell are so diverse, such agents, including NO itself, are called reactive nitrogen species (RNS), and their detrimental effects on a cell, tissue or organism is referred to as nitrosative stress (Ridnour, Thomas et al. 2004).

As RNS are diverse in their structures, they also have diverse reactivity with biological molecules (Figure 1). Dinitrogen trioxide (N_2O_3) and NO^+ can nitrosate thiols and amines. Reversible nitrosation of thiol-containing proteins can affect their activity, protein-protein interactions and cellular location, while nitrosation of amines can lead to deamination. Nitrosation and deamination can also alter the composition of DNA by converting cytosine to uracil, guanine to xanthine, methylcytosine to thymine and adenine to hypoxanthine (Pacher, Beckman et al. 2007). Peroxynitrite is an especially reactive oxidant and nitrating agent. As an oxidant it interacts directly with electron-rich groups including sulfhydryls, iron-sulfur centers, zinc-thiolates and the active sites of tyrosine

phosphatases (Beckman 1990). $ONOO^-$ can also nitrate the aromatic amino acids tyrosine and tryptophan, as well as lipids (Pacher, Beckman et al. 2007).

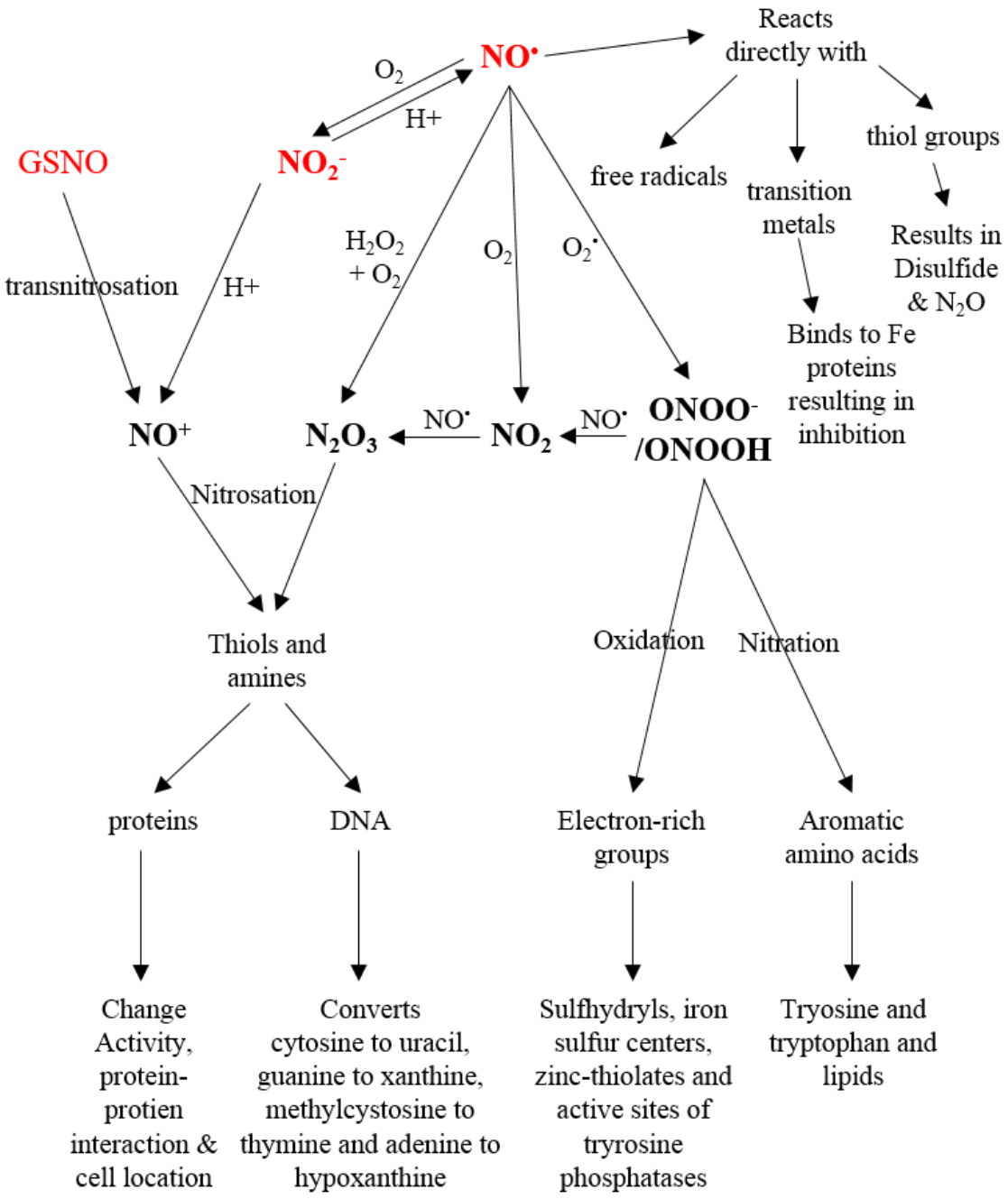


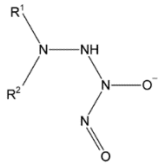
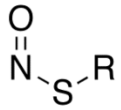
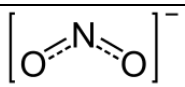
Figure 1: Nitric Oxide Chemistry (RNS) in biological systems. In **BOLD** is the chemical species that is produced and in RED are the RNS species used in this thesis.

The RNS described above arise from the reactions of NO with oxygen. NO can also react directly with thiol groups, transition metals (particularly the iron of heme and iron-sulfur clusters) and free radicals. NO reacts with thiols to produce disulfide and nitrous oxide (N_2O) (DeMaster, Quast et al. 1995). Reactions with heme iron are especially important, as both ferric heme (Fe^{3+}) and ferrous heme (Fe^{2+}) can bind NO as a ligand with high affinity, and the affinity of many heme proteins for NO exceeds that of both oxygen and carbon monoxide. As a result, heme proteins that bind exogenous ligands as part of their function, such as hemoglobin, cytochrome *c* oxidase, and cytochrome P450 are susceptible to NO inhibition. The impact of this type of interaction on Giardia may be minimal as it lacks all of these heme proteins. However, Giardia does contain iron-sulphur cluster proteins and when modified by NO these become inactivated, protein bound-dinitrosyl iron complexes (Cooper 1999, Yang, Rogers et al. 2002).

1.4 Reactive Nitrogen Species Donor Compounds

The biological effects of NO are significant and the associated literature vast, but most experiments in this field do not use nitric oxide directly as NO gas is highly reactive, has low solubility in aqueous solutions, and can generate a variety of different RNS once introduced into an experiment. Furthermore, culturing cells with the necessary gas-handling apparatus is cumbersome. To circumvent these problems, reactive nitrogen species donor compounds are commonly used in experiments. These are water-soluble agents that release specific types of RNS and in many cases have predictable kinetics of release. The major classes used in this thesis are S-nitrosothiols, NONOates, and nitrite (Table 1) (Aga and Hughes 2008).

Table 1*:* Classes of RNS donor compounds used in this work. **BOLD** indicates the predominate RNS released.

Class	Structure	RNS Released	Pro	Con
NONOates		**NO**	Directly produces NO Predictable kinetics of release	Expensive (several $ per mg)
S- nitrosothiols		**NO^+** and NO	Easy to prepare in lab	
Nitrite		NO and NO^+	Inexpensive (pennies per g)	Requires high concentrations (20 mM)

NONOates are synthesized by reacting NO with a variety of nucleophiles. They are stable as solids but can decompose in solution giving up to 2 mols of NO per mole of reactant. The major advantage of this class is their wide range of NO release kinetics, from minutes to hours depending on the compound. For example, diethyltriamine (DETA) NONOate has the longest half-life of 21 hours at 37^0C, which makes it ideal for cell biology experiments that last for several days. The major drawback of such compounds is their expense.

S-nitrosothiols have the general structure R-SNO, and one of the most commonly used member of this class is S-nitrosoglutathione (GSNO). Glutathione is a tripeptide (γ-Glu-Cys-Gly). The major pathway for GSNO is as a transnitrosating agent rather than a NO donor, providing that there are strong nucleophilic species present to act as receptors of the NO^+ group. They can decompose in solution to generate NO and RS radicals by homolytic decomposition, but this reaction is slow and therefore is not likely a major

pathway for S-nitrosothiol decomposition. Thus GSNO does not have a single mode of action in biological systems (Spiro 2006).

Nitrite (NO_2^-) is a by-product of the reaction of NO with O_2 and under mildly acidic conditions will produce NO and NO^+. Although not as precise a reagent as either the nitrosothiols or NONOates, sodium nitrite is relatively stable and inexpensive (Ridnour, Thomas et al. 2004, Spiro 2006, Aga and Hughes 2008).

1.5 Flavohemoglobin

Reactive nitrogen species are encountered by microorganisms through their own metabolic pathways or in their environment. Flavohemoglobin is an enzyme that converts NO to a stable nitrate (NO_3^-) decreasing the potential toxicity to the cell by several orders of magnitude (Forrester and Foster 2012). Flavohemoglobin is commonly found in Gram-negative bacteria, but it also has been found in some fungi and protozoan parasites, including Giardia (Rafferty and Dayer 2015).

Interestingly, the discovery of flavohemoglobin preceded the identification of its role by several decades. The first purification and characterization of flavohemoglobin was completed by Chance and colleagues in 1971 (Oshino, Asakura et al. 1972, Oshino, Asakura et al. 1973), who isolated a 50 kDa protein from the yeast *Candida mycoderma* that contained equimolar amounts of heme and flavin adenine dinucleotide (FAD). The enzyme underwent NAD(P)H-dependent reduction of heme from the ferric (Fe^{3+}) to ferrous (Fe^{2+}) states and by doing so was able to bind oxygen (Oshino, Asakura et al. 1973). Similar proteins were found in the Gram-negative bacteria *Alcaligenes eutrophus* and *E. coli*, extending the presence of flavohemoglobin to two kingdoms (Vasudevan, Armarego et al. 1991, Probst, Wolf et al. 1979). Since then, flavohemoglobins have been

identified in the genomes of other yeast species such as *Saccharomyces cerevisiae* and *Candida norvegensis*, in other Gram-negative enteric bacteria, Gram-positive bacteria and at least two protists (Iwaasa, Takagi et al. 1992, Zhu and Riggs 1992). A role for flavohemoglobins was finally determined in the late 20th century when it was found that flavohemoglobin expression in *E. coli* and *S. enterica* increased when exposed to an exogenous source of NO and was correlated with a cyanide-sensitive NO-consuming enzyme (Poole, Anjum et al. 1996, Crawford and Goldberg 1998, Gardner, Costantino et al. 1998).

Flavohemoglobin is a fusion protein consisting of an amino-terminal, heme-binding globin domain, and a carboxy-terminal FAD and nicotinamide binding domain that is a member of the ferredoxin: $NADP^+$ reductase (FNR) family. The globin domain is similar in tertiary structure to those of the oxygen transporting animal globins but their sequence similarity is low. Rather, the globin domain sequence of the bacterial flavohemoglobins more closely resembles those of the single-domain bacterial globins such as that of *Vitreoscilla* hemoglobin, the first discovered bacterial hemoglobin (Vasudevan, Armarego et al. 1991).

Flavohemoglobin evolved approximately 2 billion years ago in bacteria with the fusion between FNR and globin domains (Vinogradov, Hoogewijs et al. 2007). It has been suggested that before this time, prior to photosynthesis and an aerobic atmosphere, that NO, NO_2^- and NO_3^-, rather than O_2, were terminal electron acceptors of bacterial respiratory chains due to their high abundance from exothermic geological reactions (Ducluzeau, van Lis et al. 2009). With the rise of O_2 levels, bacteria would be under pressure to convert ATP-generating NO reductases to more efficient O_2 reductases (van

der Oost, de Boer et al. 1994, Hendriks, Gohlke et al. 1998). As these O_2 reductases were probably inhibited by NO in the atmosphere there was likely selective pressure to evolve NO-consuming enzymes such as flavohemoglobin.

Unlike bacterial flavohemoglobins, the eukaryotic isotypes are used to protect from NO produced by host immune systems (Forrester and Foster 2012). Eukaryotic flavohemoglobins probably originated through horizontal gene transfer from a bacterial source. In the case of Giardia, it likely acquired its flavohemoglobin gene from an enterobacteriaceae source, as sequence identity to these is high (40%) and both are found within the gastrointestinal tract of similar hosts. In contrast, the Giardia flavohemoglobin is much more divergent from fungal isotypes with approximately 25% identity to these proteins. Owing to the high level of sequence similarity between the *E. coli* flavohemoglobin (called Hmp) and Giardia flavohemoglobin (gFlHb) the known structure of the former can be used as a template to generate a homology model structure of the latter, using programs such as I-TASSER and SWISS-MODEL (Figure 2 & 3).

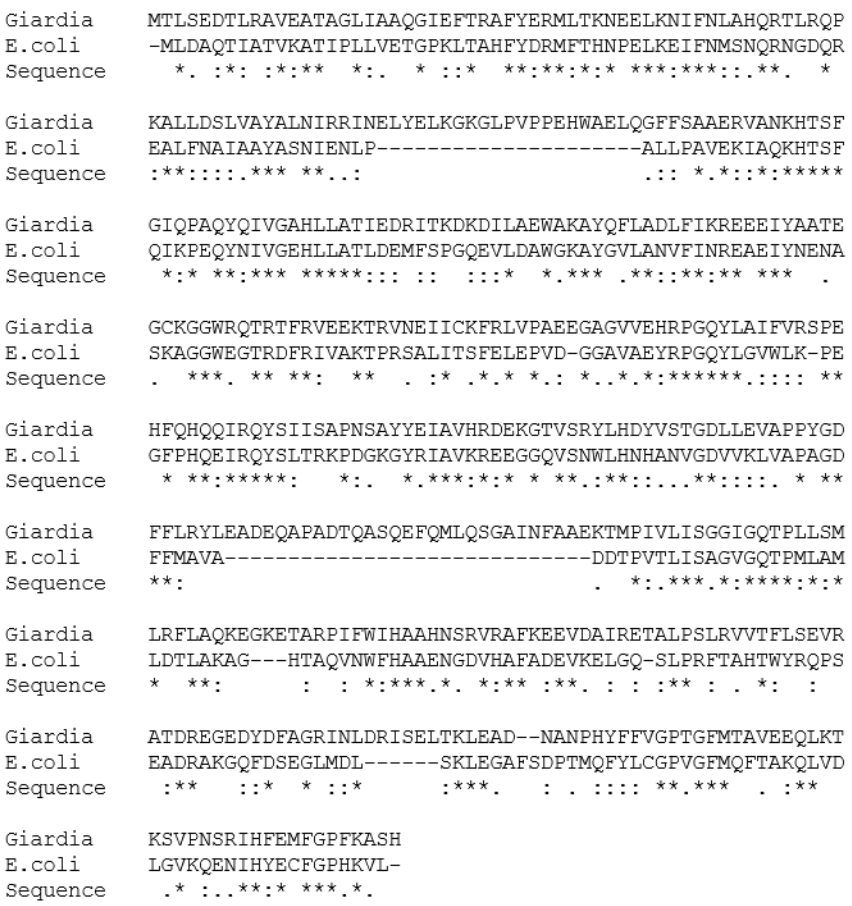


Figure 2: Sequence comparison between *Giardia intestinalis* gFlHb (UniprotKB accession number E2RTZ4) and *E. coli* Hmp (UniprotKB accession number P24232).

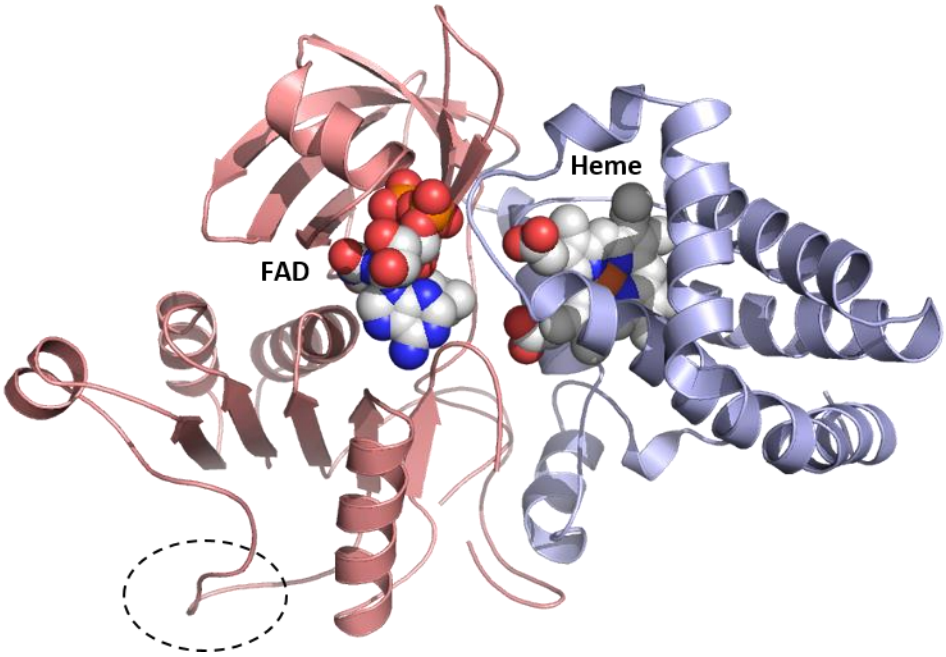


Figure 3: Homology model of gFlHb, constructed using the program SWISS-MODEL and *E. coli* Hmp as the template. The **RED** is the FNR domain and the **BLUE** is the globin domain. The location of the peptide epitope used to raise the polyclonal antibody against the protein is highlighted by the **BLACK** dotted circle.

The net reaction catalyzed by flavohemoglobin converts nitric oxide to nitrate, with oxygen and an electron as co-substrates:

$$NO + O_2 + Fe^{2+} \rightarrow NO_3^- + Fe^{3+} \quad (1)$$

These reactions occur at the heme iron, which is reduced back to the Fe^{2+} state by an electron provide by NAD(P)H *via* the FNR domain to begin another catalytic cycle. Flavohemoglobin also possesses NAD(P)H oxidase activity in which O_2 is reduced to water in the absence of NO. However, this activity is low, and Giardia has two highly active proteins (flavodiiron protein and NADH oxidase) for the purpose of O_2 detoxification (Forrester and Foster 2012, Mastronicola, Falabella et al. 2015).

Although the stoichiometry of the reaction catalyzed by flavohemoglobin is known there is some uncertainty about the mechanism, as both oxygen and nitric oxide can bind to the heme iron: oxygen to ferrous heme (Fe^{2+}), nitric oxide to either ferrous heme or ferric heme (Fe^{3+}). Depending on which binds first there are two possible mechanisms. In the *NO-dioxygenation mechanism* (Figure 4a)**,** O_2 first binds to ferrous heme in flavoHb, forming a ferrous-oxy heme complex. This reacts with NO to form a transient ferric-$ONOO^-$ intermediate, which undergoes rapid isomerization to nitrate, NO_3^-. Peroxynitrite ($ONOO^-$) is highly reactive and cytotoxic, and it is important that the isomerization to NO_3^- occurs before $ONOO^-$ can be released (Hausladen, Gow et al. 1998). The carboxy-terminal domain would then supply an electron to reduce the ferric heme for the next catalytic cycle (Forrester and Foster 2012).

The second proposed mechanism (Figure 4b) is *O_2-nitrosylation* in which flavohemoglobin first binds to NO to form a ferrous-nitrosyl species (Fe^{2+}-NO) that then

reacts with O_2 to form nitrate (Hausladen, Gow et al. 2001). Flavohemoglobin has a much higher affinity for NO than for O_2, with Michaelis constants (K_m) of 0.2 μM and 100 μM respectively, which suggests that under certain conditions O_2-nitrosylation could be the predominant reaction under physiological conditions (Gardner, Martin et al. 2000). The ferrous-NO complex undergoes internal electron transfer to generate a ferric nitroxide (NO^-) complex which would react with O_2 to form NO_3^-. As in the first mechanism the carboxy-terminal domain would then supply the electron to reduce the ferric heme (Forrester and Foster 2012).

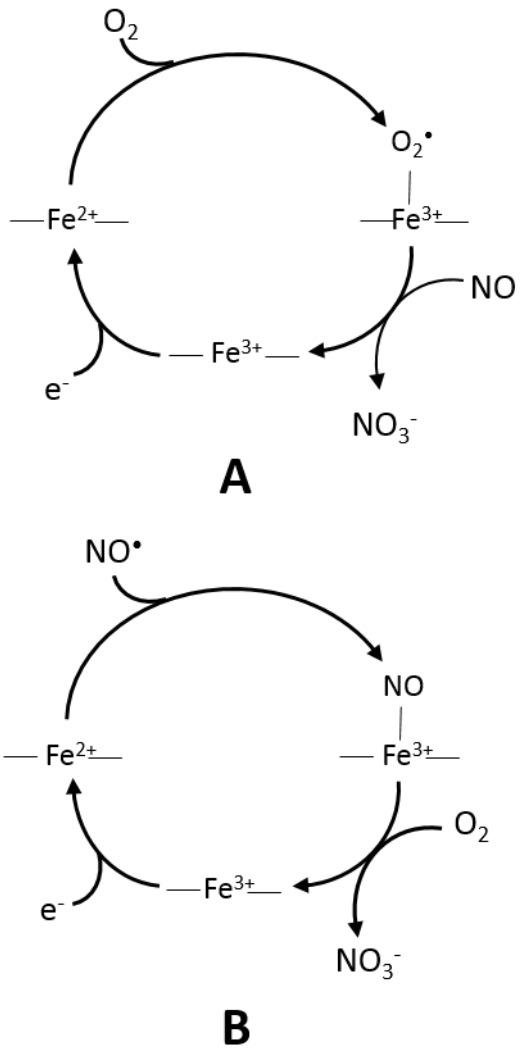


Figure 4: Proposed mechanisms of flavohemoglobin conversion of NO to nitrate. **A)** NO-dioxygenation mechanism **B)** O_2-nitrosylation mechanism

In general, flavohemoglobin is expressed only when the organism is exposed to nitrosative stress. The factors which control gene expression of flavohemoglobin are well studied in bacteria, somewhat understood in yeast but not understood in protozoan parasites such as Giardia. In both bacteria and yeast flavohemoglobin expression is controlled at the transcriptional level (Table 2).

Table 2*:* Transcription Factors which control the expression of flavohemoglobin genes. ISC, iron-sulfur cluster; ZIF, zinc finger.

Transcription factor	Organism	Effect	Family
Bacterial			
ArnR	*Corynebacterium glutamincum*	Repressor	ISC
ResDE (two proteins)	*Bacillus subtilis*	Activator	ResDE family
NorR	*Pseudomonas aeruginosa*	Activator	AAA+ (Fe^{2+} binding protein)
NsrR	Gram- and Gram+	Repressor	Rrf2 family (binds ISC)
Fungal			
Fzf1p	*Saccharomyces cerevisiae*	Activator	ZIF
Cta4	*Candida albicans*	Activator	Zn_2Cys_6 ZIF
Yap7	*Candida glabrata* & *S. cervisiae*	Repressor	Basic leucine zipper (Cys_2His_2 ZIF)

Among bacteria, several of the transcription factors which are known to control NO detoxification are also iron-containing transcription factors including those with iron-sulfur cluster (ISCs). These can form nitrosyl iron complexes that lead to conformational changes in the protein, thereby changing the DNA affinity of these transcriptional factors (Fleischhacker and Kiley 2011). Examples of such transcription factors known to control flavohemoglobin gene expression include ArnR, NsrR and NorR. Interestingly this class includes both transcriptional activators and repressors. In *C. glutamincum*, ArnR has the characteristic spectrum of an iron-sulphur cluster protein (broad peak at 429 nm, shoulder at 320 nm), and in the presence of RNS donors the flavohemoglobin gene was

derepressed (Nishimura, Teramoto et al. 2014). Similarly, the ISC-containing transcription factor NsrR derepresses flavohemoglobin gene expression in a variety of Gram-positive and Gram-negative bacteria under nitrosative stress conditions (Fleischhacker and Kiley 2011). On the other hand, in *P. aeruginosa* NorR activates expression of the flavohemoglobin gene when bound to the promoter by formation of a mono-nitrosyl complex at the iron molecule at NorR N-terminus (Spiro 2007, Fleischhacker and Kiley 2011). NorR also activates other genes involved in nitrogen metabolism in other organisms, including flavorubredoxin in *E. coli* and norAB genes that encode an NO reductase in *Ralstonia eutropha* (Spiro 2006, Spiro 2007, Fleischhacker and Kiley 2011). In *Bacillus subtilis*, flavohemoglobin expression is activated by the two-component ResDE system. Here the presence of NO causes phosphorylation of ResE which causes a cascade phosphorylation of ResD to activate transcription of the flavohemoglobin gene. This system works in tandem with NsrR (Kommineni, Yukl et al. 2010).

Among eukaryotes most if not all work on the regulation of flavohemoglobin gene expression involves studies in yeast. Here the DNA-binding domains of the transcription factors are zinc fingers. Unlike bacteria the presumed RNS-sensing domains do not contain iron. Precisely how nitrosative stress is detected by these proteins is unknown but they may use cysteine residues that react with NO or NO^+. As in the case of bacteria these transcription factors include both activators (*S. cerevisiae* Fzf1p, *C. albicans* Cta4) and repressors (*S. cerevisiae and C. glabarata* Yap7) of flavohemoglobin gene expression (Sarver and DeRisi 2005, Chiranand, McLeod et al. 2008, Merhej, Delaveau et al. 2015).

Flavohemoglobin expression in Giardia trophozoites also responds to exposure to nitrosative stress. Mastronicola *et. al.* exposed Giardia trophozoites to DETA-NONOate, GSNO and nitrite (NO_2^-) for 24 hours and observed an increase gFlHb protein expression (2010). Expression appears to be controlled at the transcriptional level as gFlHb mRNA increases 4-6 fold after 12-24 h exposure to nitrosative stress (Yee *et. al.*, in preparation). Beyond these observations little is known about gFlHb expression at shorter exposure times, nor about the subcellular localization of gFlHb within the Giardia trophozoites.

1.6 Thesis Aims

The goal of this work is to use a polyclonal antibody raised against gFlHb to study its expression and location within Giardia trophozoites, and how these may change in response to nitrosative stress. This work has three parts. First, to optimize conditions for Western blotting to accomplish part 2, as this type of work had not been done in the lab before. Second, to confirm the observations of Mastronicola and coworkers that gFlHb expression increases after 24-hour exposure to RNS donor compounds (NO_2^-, GSNO, DETA-NONOate), and to extend this by doing time course experiments. Finally, immunofluorescence microscopy is used to determine the localization of gFlHb in Giardia trophozoites before and after treatment with the RNS donors NO_2^- and GSNO. The work in this thesis describes the method development necessary to achieve the aims and is a guide for future research in this area.

PROCEDURES

2.1 Culturing of *Giardia intestinalis* trophozoites

All culturing of Giardia was done within a Class 2 biosafety cabinet. *Giardia intestinalis* trophozoites strain WB, assemblage A (ATCC 50803) were cultured in modified TYI-S-33 media at 37°C in 16 mL glass culture tubes (Keister 1983). Modified TYI-S-33 media contained 0.05 g/mL yeast extract (Bioshop), 0.05 g/mL of D-glucose (Bioshop), 0.01 g/mL NaCl, 0.1 g/mL N-Z Case Plus (Fluka Analytical), 2.28 mg/mL of ferric ammonia citrate (Sigma), 10% cosmic calf serum (HyClone), 0.65 mg/mL bovine bile and 0.5 M phosphate buffer, pH 7.2. This media was filtered through a 0.45 μm filter (Thermo Scientific Nalgene rapid-flow). The media was also supplemented with sterile-filtered 50X L-cysteine/ascorbic acid and 100X antibiotic/antimycotic (HyClone). Trophozoites were transferred into fresh media when cells became confluent. This was done by chilling the culture tubes, which releases adhering trophozoites, followed by centrifugation (Beckman Coulter Allegra X14R centrifuge, 1,200 *g*, 15 minutes, 4°C) and using the cell pellet to inoculate fresh media and tubes.

2.2 RNS Donor Preparation and Use

Three reactive nitrogen species (RNS) donors were used in this work: sodium nitrite ($NaNO_2$), S-nitrosoglutathione (GSNO), and DETA-NONOate.

The following procedures were common to experiments involving all three of these reagents. Stock solutions of these reagents were sterile-filtered through a 0.2 micron filter (Sarstedt) before their addition to cultures of Giardia trophozoites, which were grown to confluency at the time of treatment. Untreated control cells were prepared by adding an equal volume of solution (water for $NaNO_2$ and GSNO negative controls; 10

mM NaOH for DETA-NONOate negative control) that did not contain the RNS donor. Giardia trophozoites were grown for up to 24 hours at 37°C, then pelleted by centrifugation as described above. When estimation of Giardia trophozoite culture cell counts were needed, cell counts were performed on a Bechman Vi-CELL XR cell counter. The media was discarded and the cell pellets were stored at -80°C.

Stock solutions of $NaNO_2$ (Sigma) were made at a concentration of 1 M in water. Sufficient stock solution was added to cultures of Giardia trophozoites to bring the concentration of $NaNO_2$ per tube to 20 mM.

GSNO was synthesized based on the protocol by Hart in which nitrous acid reacts with glutathione (GSH) (1985):

$$HNO_2 + GSH \rightarrow GSNO + H_2O \qquad (2)$$

As NO is a potent vasodilator the reaction was performed within a fumehood. A 50 mL conical tube was covered with aluminum foil to minimize light exposure and a magnetic stir rod was placed inside. Millipore-grade water (5.9 mL) was added to the tube, followed by sufficient concentrated HCl to bring to a final concentration to 3.15%. To this was added, with stirring, 0.236 g glutathione (Bioshop) and 1 mL of 0.346 g/mL sodium nitrite (Sigma). During this time the solution turned a cranberry red color as GSNO was produced. The solution was left stirring for another 5 minutes to allow the reaction to complete. The pH was adjusted to 7.0 by the addition of 95% NaOH equivalent to the reaction and the volume was adjusted to 10 mL with Millipore-grade water. The concentration of GSNO was determined spectrophotometrically on a Cary 400 Bio spectrophotometer at 335 nm using the Beer-Lambert equation and an extinction

coefficient of 922 M^{-1} cm^{-1}. Solutions were divided into 500 μL aliquots and stored at -80°C. GSNO was added to culture tubes of Giardia trophozoites to a final concentration of 1 mM or 5 mM.

100 mM stock solutions of DETA-NONOate (Fisher Scientific/ACROS) were prepared fresh on the day of use by dissolving 20 mg in 1.2 mL of 10 mM NaOH on ice, as NONOates are stable under basic conditions and will not release NO until conditions are neutral or acidic. The 100 mM DETA-NONOate stock solution was added to the culture tubes to a final concentration of 1 mM.

2.3 Anti-gFlHb Antibody

Below is the amino acid sequence of gFlHb (giardiadb.org GL50803_15009):

MTLSEDTLRAVEATAGLIAAQGIEFTRAFYERMLTKNEELKNIFNLAHQRTLRQPKALLDSLVAYALNIRRI
NELYELKGKGLPVPPEHWAELQGFFSAAERVANKHTSFGIQPAQYQIVGAHLLATIEDRITKDKDILAEWAK
AYQFLADLFIKREEEIYAATEGCKGGWRQTRTFRVEEKTRVNEIICKFRLVPAEEGAGVVEHRPGQYLAIFV
RSPEHFQHQQIRQYSIISAPNSAYYEIAVHRDEKGTVSRYLHDYVSTGDLLEVAPPYGDFFLRYLEADEQAP
ADTQASQEFQMLQSGAINFAAEKTMPIVLISGGIGQTPLLSMLRFLAQKEGKETARPIFWIHAAHNSRVRAF
KEEVDAIRETALPSLRVVTFLSEVRATDREGEDYDFAGRINLD**RISELTKLEADNAN**PHYFFVGPTGFMTAV
EEQLKTKSVPNSRIHFEMFGPFKASH

The services of Genscript (Piscataway, NJ) were used to raise a rabbit polyclonal antibody against the peptide epitope shown UNDERLINED, above, which was predicted to be strongly immunoreactive according to their proprietary software. This corresponds to a surface-exposed loop in the C-terminal FNR domain of the protein (Figure 4).

2.4 Western Blotting

Stored cells were thawed at room temperature, resuspended in phosphate-buffered saline (PBS), and centrifuged (Beckman Coulter Microfuge 22R, 1,200 *g*, 4°C, 15 minutes). The supernatant was discarded and the cells were lysed with RIPA buffer (2.5-5 μL/mg of Giardia cells) (150 mM NaCl, 1.0% NP-40 0.5% sodium deoxycholate, 0.1%

SDS and 50 mM Tris, pH 8.0) containing the protease inhibitor leupeptin to a final concentration of 10 μg/mL. The solution was incubated in a 1.5 mL microfuge tube with gentle agitation on a rotator at 4°C for 30 to 60 minutes, followed by centrifugation at 10,000 *g*, 4°C, 10 minutes. The supernatant was collected and the pellet was discarded. Protein concentrations of the supernatants were determined spectrophotometrically by Bradford Assay (Bioshop Canada) on a Shimadzu UV-160 spectrophotometer at a wavelength of 595 nm, using bovine serum albumin protein standards (Thermo-Fisher/Pierce).

Protein samples (typically 40 μg per lane) and a prestained ladder (NEB #P07703 or #P07706) were loaded onto 10% SDS-PAGE gels and electrophoresed at 80 V for 15 minutes, followed by 150 V for an hour. For transfer of proteins from the gel to the nitrocellulose membrane, the gel, with a 0.2 μm nitrocellulose membrane (Amersham: 10600044) and 6 pieces of Whatman paper were incubated for 10 minutes in Towbin buffer (25 mM of Tris, 192 mM glycine, 20% methanol). These were then assembled into the sandwich on a semi-dry transfer apparatus (OWL-HEP-1) to transfer the proteins from the gel to the membrane. The proteins were transferred for 90 minutes at a current of 100 mA, a voltage of 14 V, and a power setting of 200 W.

After the transfer was complete, membranes were treated with a Ponceau stain (Bioshop Canada) to verify a successful transfer and confirm equal protein loading across all lanes. Membranes were rinsed with deionized water and pictures were taken. To remove the excess stain, the membrane was incubated with 0.1% Tween in PBS for 5 minutes followed by blocking with 5% skim milk powder in PBS for 1 hour at room temperature, or overnight incubation at 4°C on a rocker.

Membranes were incubated at room temperature for 2 hours on a rocker with either a 3,000-fold dilution of anti-gFlHb antibody in 5% BSA, 0.01% Tween 20 in PBS, or a 10,000-fold dilution in 5% skim milk powder, 0.01% Tween 20 in PBS. Following incubation with the primary antibody, membranes were briefly rinsed three times in 0.1% Tween 20 in PBS, followed by three five-minute washes in the same buffer at room temperature on a rocker.

Membranes were subsequently treated with a goat anti-rabbit horseradish peroxidase conjugate (GAR-HRP) as the secondary antibody (Jackson ImmunoResearch: 115-035-003). Each membrane was incubated for one hour with GAR-HRP diluted 5,000-fold in PBS containing 5% skim milk powder and 0.01% Tween 20 at room temperature on a rocker. Excess secondary antibody was washed off as described for the primary antibody.

Immunoreactive bands were detected by chemiluminescence with either PerkinElmer Western Lightning ECL Pro, or Bio-Rad Clarity ECL Western Blotting substrate. Images were captured and stored on a Syngene Chemi Genius2 Bio Imaging system, which was later replaced with a Bio-Rad Chemi Doc MP Imaging System. In one case a blot was developed using the HRP-chromogenic reagent, tetramethylbenzidine (1-Step™ TMB-Blotting Substrate Solution; Thermo-Fisher, Pierce). To normalize gFlHb protein expression the program ImageJ (http://imagej.net/) was used and in which the images were desaturated and inverted, followed by measuring the integrated intensities. The total protein intensities were used to calculate the normalization factor (no-exposed intensity/ time point intensity) and was multiplied by the corresponding gFlHb intensity to give the relative intensity after normalization. Fold changes were calculated by taking

the normalized relative intensity of a given time point and dividing it by the untreated sample. In some experiments (Figures 8, 9 and 15), a 52 kDa band corresponding to gFlHb was not observed due to its low abundance in untreated cells and the short exposure time used for the capture of the chemiluminescent signal from the Western blots. As a result, the normalization of the gFlHb protein levels, and hence, the calculated fold changes in these levels may not be accurate. The Western blot analysis should be repeated in these experiments so that a band corresponding to the 52 kDa gFlHb band could be observed in the untreated protein samples to allow for a more accurate determination of the fold-changes in the gFlHb level. In future experiments, it is recommended that twice the amount of protein for the untreated sample should be loaded into the gel so that the 52 kDa band could be seen in the Western blot without the need to increase the exposure time, which may saturate the signal intensity of this band in the samples from the other time points.

2.5 Immunofluorescence Microscopy

Giardia trophozoites were exposed to either 5 mM GSNO or 20 mM $NaNO_2$ for 24 hours while untreated cells were grown for the same period. Cells were collected by centrifugation twice at 1,000 *g* for 10 minutes at 4°C (Beckman Coulter Allegra X14R centrifuge or Beckman Coulter Microfiber 22R centrifuge). Cells were resuspended in 1xPBS and allowed to adhere to microscope slide cover slips pre-treated with 0.1% polyethylenimine for 20 minutes at 37°C. Cells were fixed by placing the cover slips in prechilled methanol for 10 minutes at -20°C, and were air dried for 5 minutes at room temperature.

The remaining steps from this point forward were performed at room temperature and on parafilm-lined trays. The trophozoites were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes and followed by a one-hour incubation in blocking buffer (5% BSA, 50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 0.5% NP-40). The slides were then incubated in 1,000-fold dilution of anti-gFlHb polyclonal antibody in blocking buffer for 1 hour and washed 4 times with PBS for 5 minutes. For treatment of slides with the secondary antibody, slides were incubated with a 200-fold dilution of GAR conjugated to the fluorescent dye Cy3 (Jackson Immuno Research) in blocking buffer for 1 hour, and then washed 4 times with PBS for 5 minutes. The cover slips were treated with 3.7% paraformaldehyde in PBS for 10 minutes to post-fix the cells, washed twice with PBS for 5 minutes and then once with water for 5 minutes. The cover slips were mounted to slides with Vectashield mounting medium containing the fluorescent DNA stain DAPI, sealed with clear nail polish and left to cure overnight. Slides were visualized with a Leica DM 6000B epifluorescent microscope at 1,600X oil magnification, 100X oil objective lens. The exposure time was 500 ms for the untreated trophozoites and for the RNS-donor treated trophozoites.

2.6 Identification of potential sumoylation sites on gFlHb

Sites on gFlHb that may act as points of covalent binding by small ubiquitin-like modifier (SUMO) protein were identified by using the SUMOplot application (http://www.abgent.com/sumoplot).

RESULTS

3.1 Method development

For Western blots the primary antibody concentration of rabbit anti-gFlHb polyclonal antibody used depended on the method of detection (chemiluminescent or chromogenic) and the chemiluminescence detection system used. During the course of this work the original gel imager, a Syngene Chemi Genius2 Bio Imaging system suffered two breakdowns and was ultimately replaced in August 2015 by a more sensitive instrument, a Bio-Rad Chemi Doc MP Imaging System. This led to a change from a 3000-fold dilution of the primary antibody in earlier experiments to 10,000-fold dilution in later experiments. Only one experiment was done with chromogenic detection, at 3,000-fold dilution of the primary antibody. The dilution of the GAR-HRP secondary antibody worked well at a 5,000-fold dilution in all cases, and was not altered. The anti-gFlHb Ab was determined to be specific by comparing the pre-immune serum to the anti-gFlHb Ab (Figure 5).

In the early Western blots an intense but nonspecific high molecular weight band around 100 kDa was observed across all lanes that contained cell lysates, regardless of whether trophozoites were treated with RNS donors or not (Figure 6). The presence of this band was alleviated by three factors. First, this band was more prominent in cell lysates that had been stored in the -80° freezer. Rather than store cell lysates, I stored cell pellets, which were then lysed just before Western blotting. Second, the intensity of the band significantly decreased if the cell lysates in SDS-PAGE loading buffer were not heated before gel electrophoresis. Finally, the protease inhibitor used in the lysates was switched from a mixture of different inhibitors (PIC- Protease Inhibitor Cocktail, Bioshop

Canada) to leupeptin (Bioshop Canada), a cysteine protease inhibitor. In Giardia the majority of proteases are cysteine proteases (DuBois, Abodeely et al. 2008). However, the 100 kDA band was found to be present after the new chemiluminescence (Bio-Rad) imaging system came, this was because the new system was found to be more sensitive than the old (Syngene) system, but was found to be still less intense than samples with heating (Figure 6). One possible explanation for the 100 kDa band is that it is a protein with a buried epitope that reacts non-specifically to the anti-gFlHb antibody and when heated this epitope is revealed by the unfolding of the protein.

In optimal conditions Giardia cultures grow with a doubling time of 6 hours for WB isolate cells. The addition of RNS donors to Giardia trophozoite cultures slowed their growth considerably and after 24-hour exposure to the RNS donors the cell density was about a third of the untreated cells. To compensate for this decrease in cells and resultant protein yield, I grew 3 tubes of RNS-treated trophozoites for every one tube of untreated control cells.

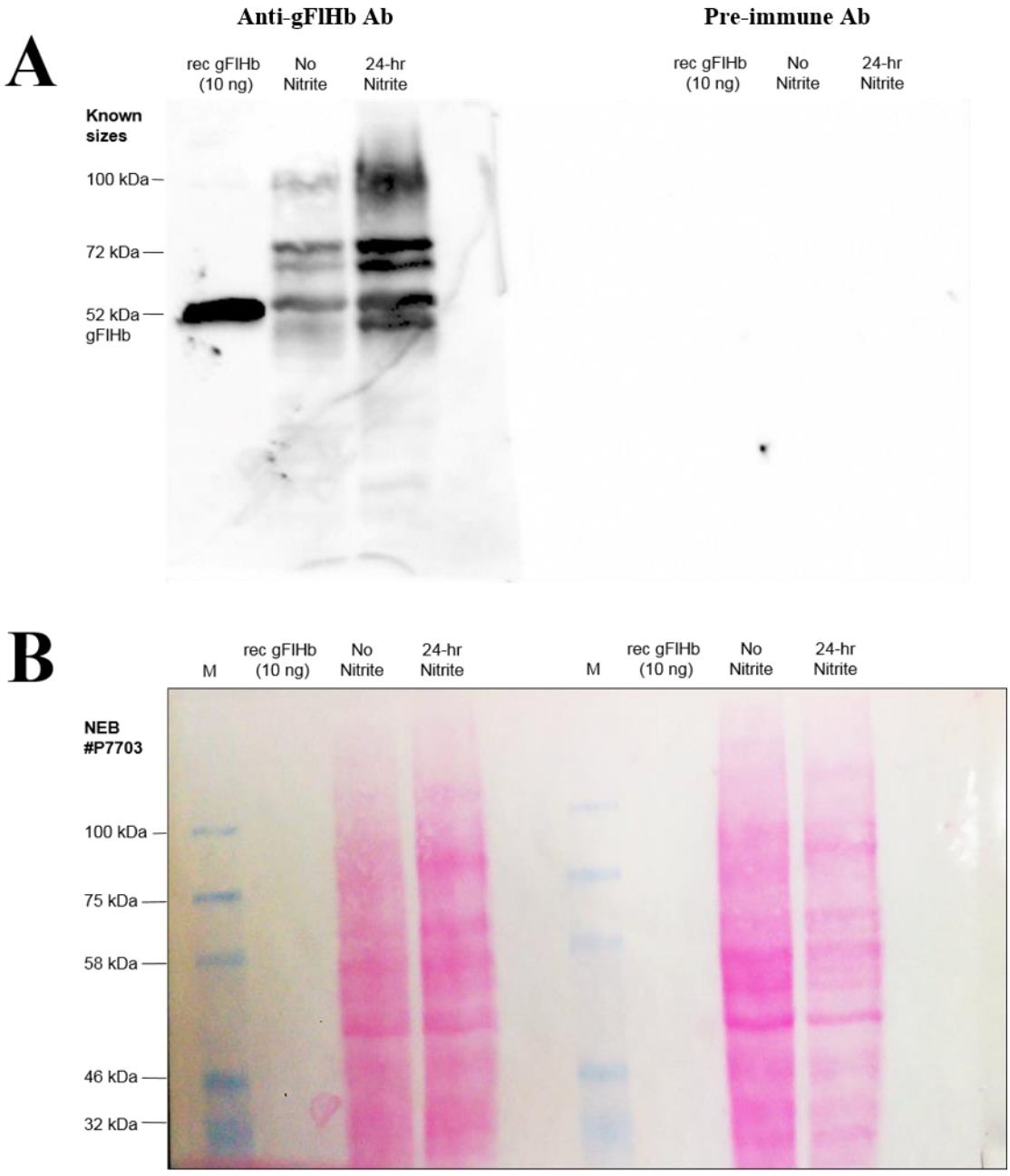


Figure 5: Western Blotting results comparing the anti-gFlHb antibody against the pre-immune serum. **A)** Western blots of Giardia trophozoite lysate probed with rabbit anti-gFlHb antibody (left) or rabbit pre-immune serum (right). Both antibody and serum were diluted 3,000 fold and tested on untreated and 20 mM nitrite exposed trophozoite lysates with chemiluminescent detection (56 µg of protein lysate was loaded in each lane). **B)** Ponceau staining of the blot.

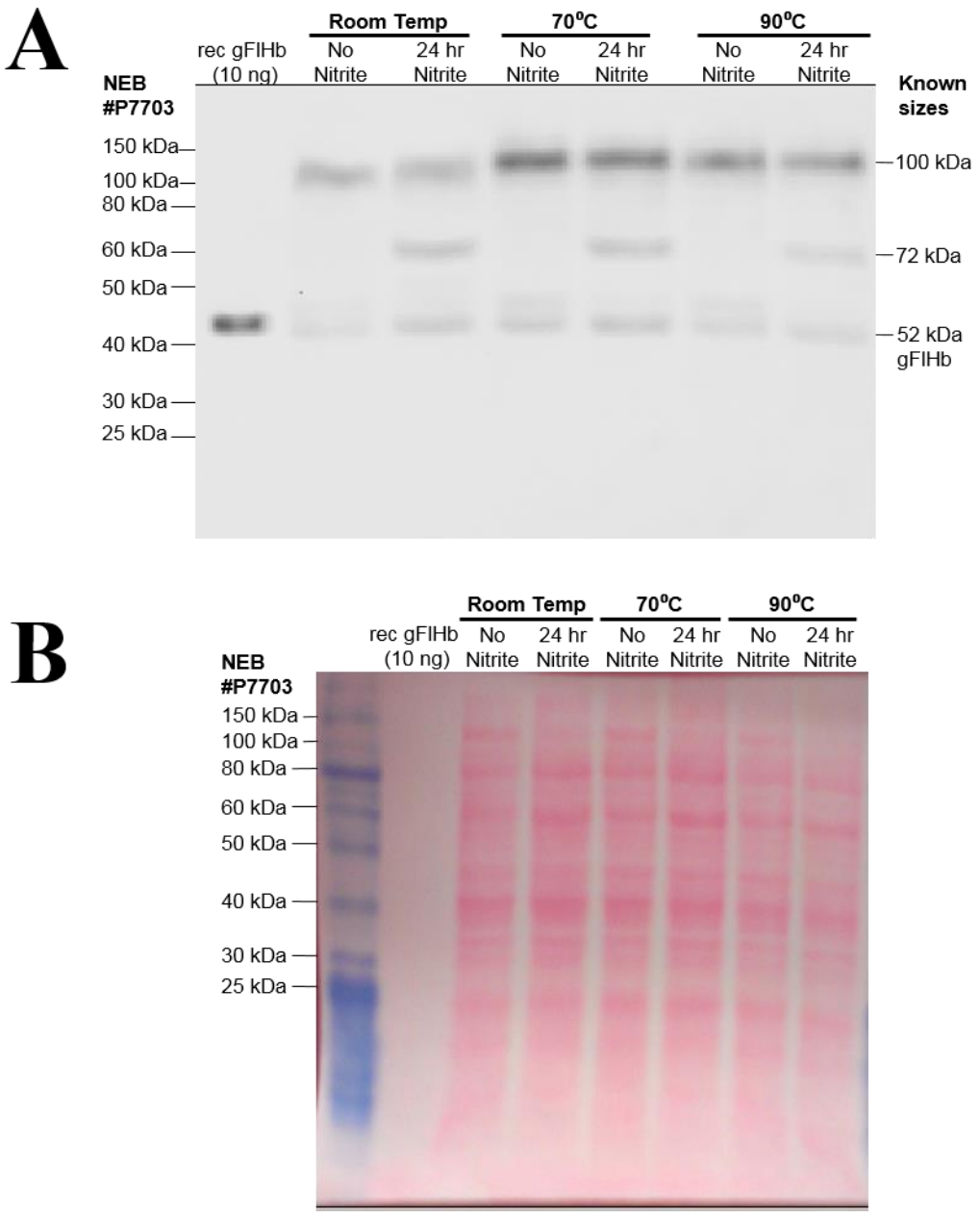
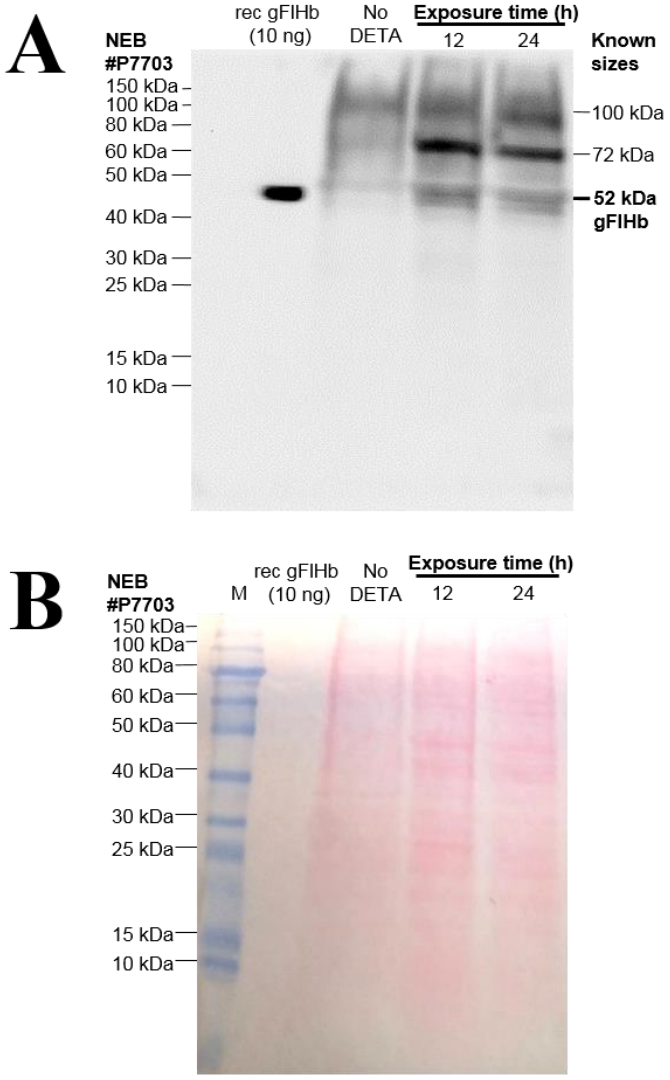


Figure 6: Changes in 100 kDa band intensity in response to heating cell lysates in SDS-PAGE loading dye **A)** Giardia cells were either not exposed or exposed to 20 mM of nitrite and then extracted protein was incubated at room temperature, 70°C or 100°C in SDS-PAGE loading dye. 25 ug of protein were loaded per lane. **B)** Ponceau staining of the blot.

3.2 Effect of 24-hr exposure to RNS donors on gFlHb expression

Giardia flavohemoglobin expression was induced by all three RNS donors (1 mM DETA-NONOate, 20 mM $NaNO_2$, 1 mM GSNO) after 24 hours, as seen by the increase in the intensity of a band near 52 kDa, which corresponds to the predicted size of gFlHb (Figures 7-9)**.** This is consistent with the observations of Mastronicola and coworkers who treated Giardia trophozoites with these RNS donors, under the similar conditions (20 mM $NaNO_2$, 2 mM DETA-NONOate, 5 mM GSNO) for 24 hours (2010).

Interestingly, I observed an additional immunoreactive band at a higher molecular weight (~72 kDa) that was present only in RNS-donor treated cells and not in untreated cells (Figures 7, 8 & 10). In some cases, this band was more intense than the 52 kDa band assigned to gFlHb. This observation, which was not reported by Mastronicola and coworkers, suggests that gFlHb is covalently modified after translation. An apparent 20 kDa increase in size on SDS-PAGE is consistent with an isopeptide bond between the C-terminal glutamic acid of small ubiquitin-like modifier protein (SUMO) and a side chain lysine of gFlHb within the consensus tetrapeptide sequence Φ-K-X-(D/E), where Φ is a hydrophobic amino acid and X is any amino acid. As it is known that Giardia expresses SUMO, I searched for sumoylation consensus sites within gFlHb using the application Sumoplot (Touz, Ropolo et al. 2008). Two high-probability sites were identified at K155 (probability = 0.94) and K361 (probability = 0.85) (Figures 11 & 12).

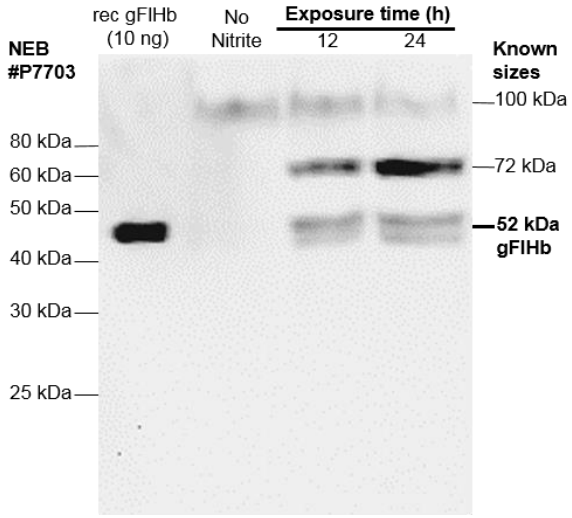


C

Hours of Exposure to DETA-NONOate	gFlHb Relative Intensity	Total Protein Relative Intensity	Normalizer Factor	Relative Intensity after Normalization	Relative to no exposure
untreated	4.818	54.207	1.00	4.82	1.00
12	8.616	45.278	1.20	10.32	2.14
24	7.804	41.700	1.30	10.14	2.11

Figure 7: Response of flavohemoglobin protein expression after Giardia exposure to DETA-NONOate. **A)** gFlHb expression in response to 1 mM DETA-NONOate with chemiluminescent detection. 30 µg protein lysate was loaded per lane **B)** Ponceau staining of the blot. **C)** Normalization of gFlHb protein expression to total protein from Ponceau staining

A



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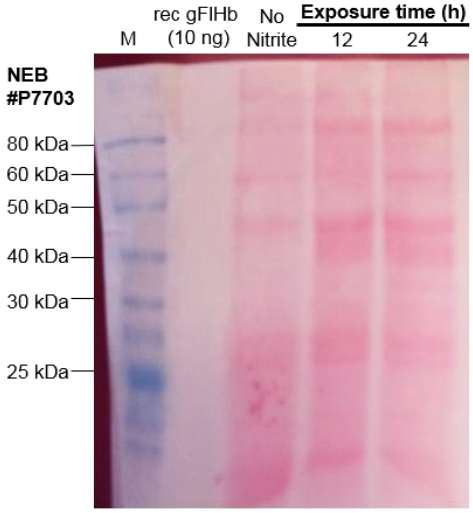
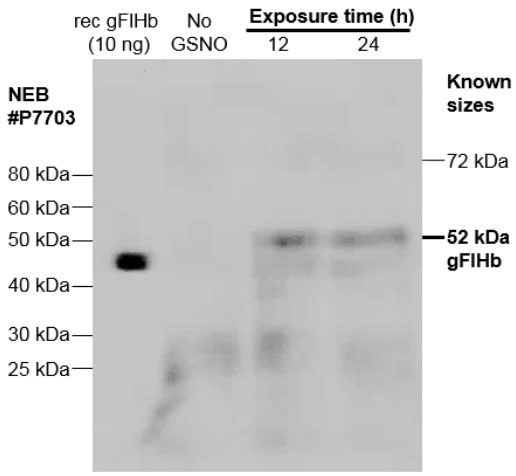


C

Hours of Exposure to Nitrite	gFlHb Relative Intensity	Total Protein Relative Intensity	Normalizer Factor	Relative Intensity after Normalization	Relative to no exposure
untreated	2.579	79.385	1.00	2.58	1.00
12	4.963	76.127	1.04	5.18	2.01
24	5.494	68.660	1.16	6.35	2.46

Figure 8: Response of flavohemoglobin protein expression after Giardia exposure to nitrite. **A)** gFlHb expression in response to 20 mM sodium nitrite with chemiluminescent detection. 50 µg of protein lysate was loaded in each lane. **B)** Ponceau staining of the blot. **C)** Normalization of gFlHb protein expression to total protein from Ponceau staining. Since the 52 kDa band for gFLHb is not visible in the untreated/no nitrite lane, it is necessary to repeat this experiment to obtain a more accurate calculation of the fold change for this protein.

A



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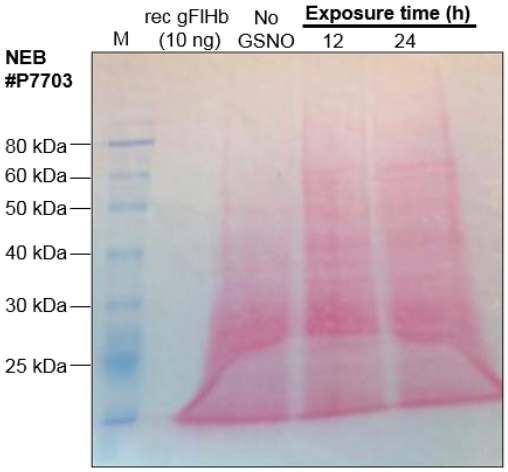


C

Hours of Exposure to GSNO	gFlHb Relative Intensity	Total Protein Relative Intensity	Normalizer Factor	Relative Intensity after Normalization	Relative to no exposure
untreated	10.222	48.288	1.00	10.22	1.00
12	11.364	49.591	0.97	11.07	1.08
24	10.928	49.458	0.98	10.67	1.04

Figure 9: Response of flavohemoglobin protein expression after Giardia exposure to 1 mM GSNO. **A)** gFlHb expression in response to 1 mM GSNO, with chemiluminescent detection. 40 µg of cell lysate was loaded per lane **B)** Ponceau staining of the same blot. **C)** Normalization of gFlHb protein expression to total protein from Ponceau staining. Since the 52 kDa band for gFLHb is not visible in the untreated/no GSNO lane, it is necessary to repeat this experiment to obtain a more accurate calculation of the fold change for this protein.

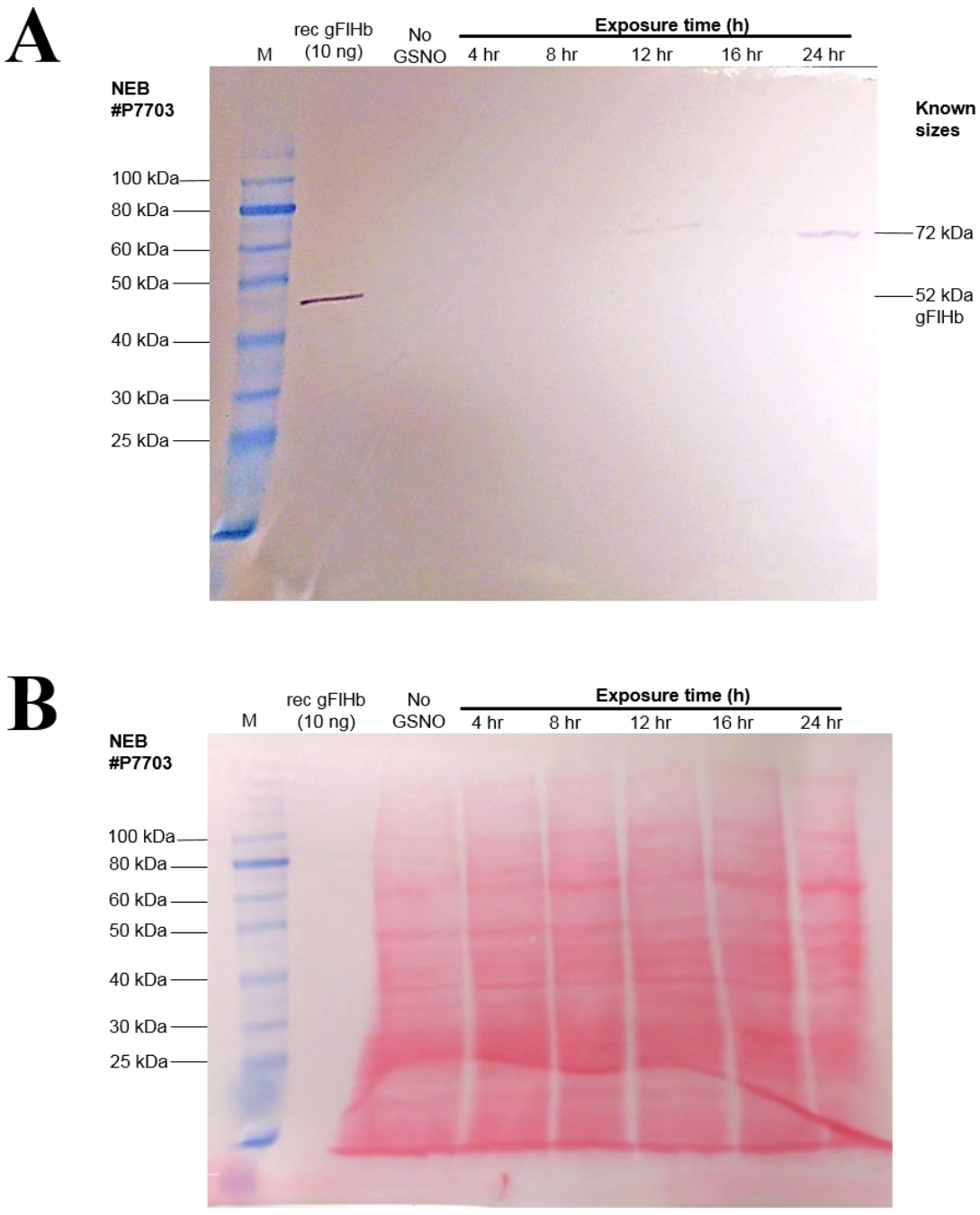


Figure 10: Response of flavohemoglobin protein expression after Giardia exposure to 5 mM GSNO. **A)** Time course of gFlHb expression in response to 5 mM GSNO, with chromogenic detection. 80 µg of cell lysate were loaded per lane. **B)** Ponceau staining of the same blot.

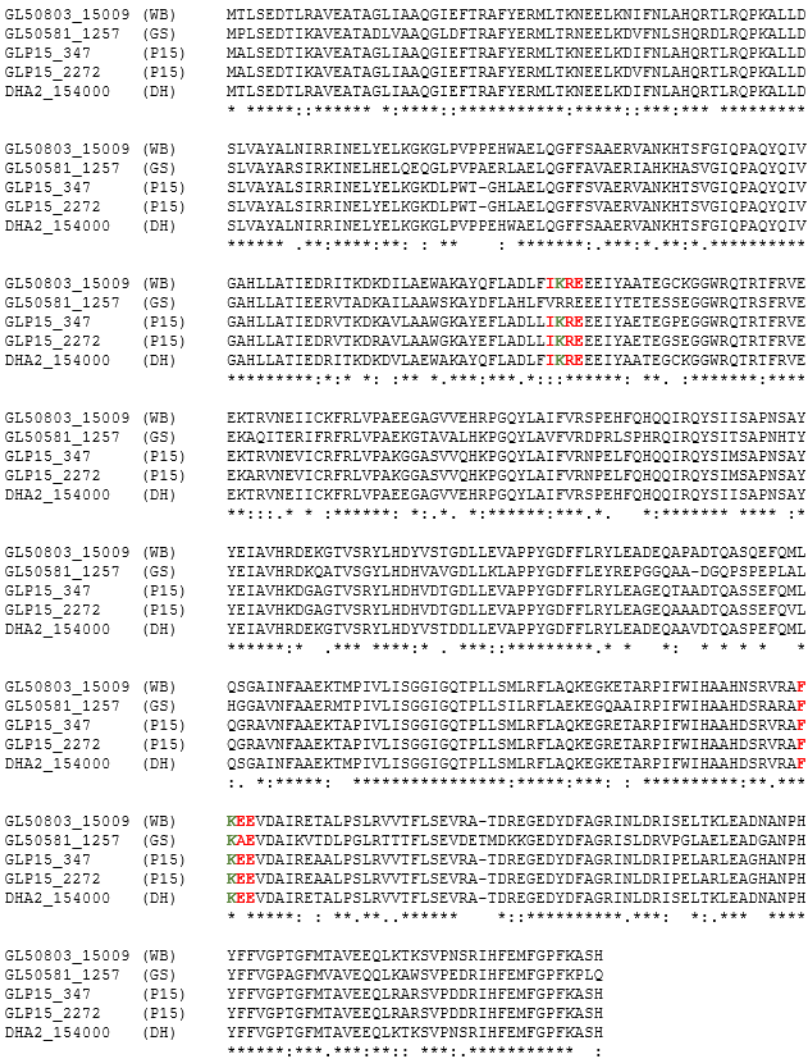


Figure 11: High-probability sumoylation sites identified in gFlHb of different Giardia assemblages in **RED**, with the lysine residues that are the sites of potential isopeptide bonds to SUMO in **GREEN**.

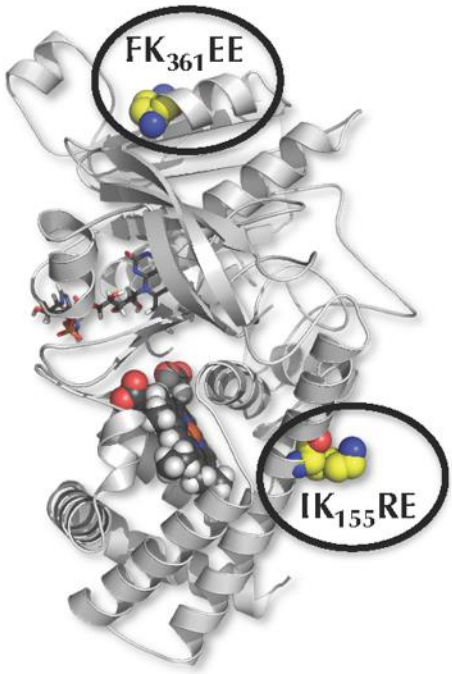
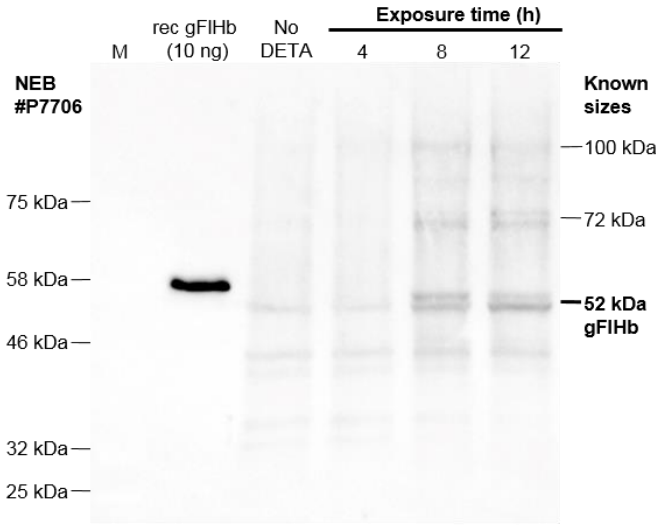


Figure 12: Potential sumoylation sites on gFlHb WB assemblage, as indicated by black circles.

3.3 Time Course of gFlHb Expression on treatment with RNS donors

From the Western blots of GSNO and DETA-NONOate treated cells it was seen that gFlHb increased gFlHb expression was detectable 4 hours after exposure for GSNO and 8 hours after exposure for DETA-NONOate, and continued to increase for the proceeding time points (Figures 13 & 14). It took longer for gFlHb to be detected in trophozoites treated with $NaNO_2$ (Figure 15), as they were seen to have increased gFlHb protein expression by 12 hours and onwards. This difference may be due to slower release of NO from sodium nitrite compared to the other RNS donors, as well as an overall lower concentration of NO.

A



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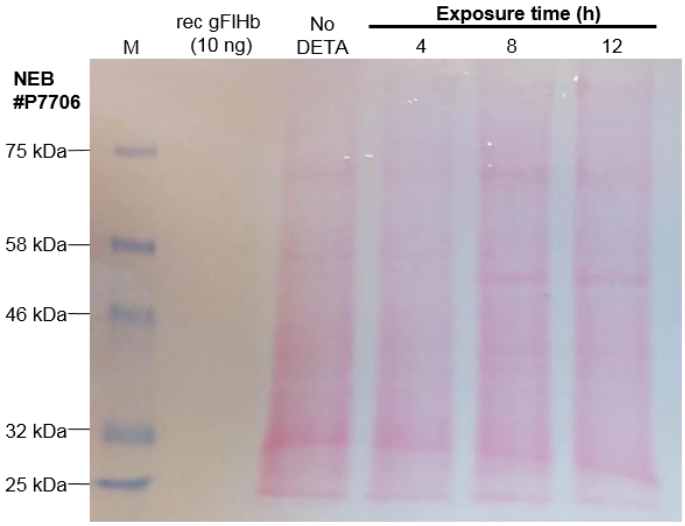
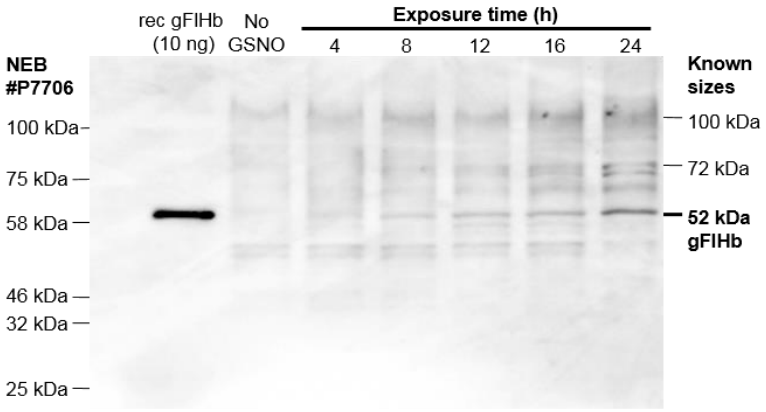


C

Hours of Exposure to DETA-NONOate	gFlHb Relative Intensity	Total Protein Relative Intensity	Normalizer Factor	Relative Intensity after Normalization	Relative to no exposure
untreated	1.305	107.517	1.00	1.31	1.00
4	1.153	98.869	1.09	1.25	0.96
8	2.857	98.440	1.09	3.12	2.39
12	3.029	95.501	1.13	3.41	2.61

Figure 13: Time course experiments of Giardia trophozoites exposed to DETA-NONOate to determine changes in gFlHb protein expression. **A)** Time course of gFlHb expression in response to 1 mM DETA-NONOate with chemiluminescent detection. 40 µg protein lysate was loaded per lane. **B)** Ponceau staining of the blot. **C)** Normalization of gFlHb protein expression to total protein from Ponceau staining

A



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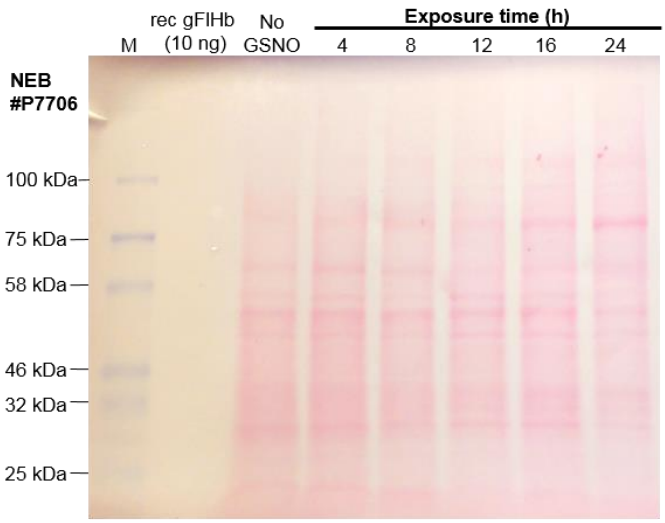
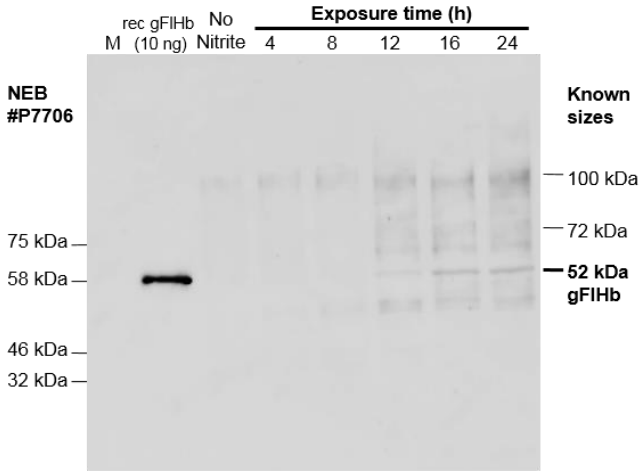


C

Hours of Exposure to GSNO	gFlHb Relative Intensity	Total Protein Relative Intensity	Normalizer Factor	Relative Intensity after Normalization	Relative to no exposure
untreated	0.902	30.944	1.00	0.90	1.00
4	1.345	29.033	1.07	1.43	1.59
8	1.324	25.439	1.22	1.61	1.79
12	1.654	22.105	1.40	2.32	2.57
16	2.015	20.413	1.52	3.05	3.39
24	3.092	19.489	1.59	4.91	5.44

Figure 14: Time course experiments of Giardia trophozoites exposed to GSNO to determine changes in gFlHb protein expression. **A)** Time course of gFlHb expression in response to 5 mM GSNO with chemiluminescent detection. 40 µg protein lysate was loaded per lane. **B)** Ponceau staining was used to determine approximately equal loading and transfer of total protein between different samples. **C)** Normalization of gFlHb protein expression to total protein from Ponceau staining

A



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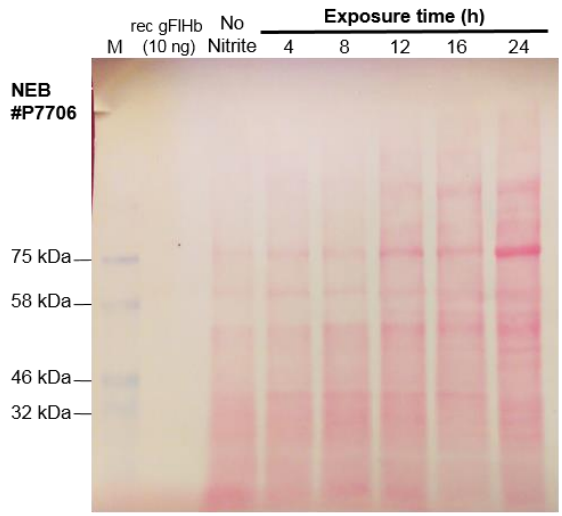

C

Hours of Exposure	gFlHb Relative Intensity	Total Protein Relative Intensity	Normalizer Factor (no intensity/ time point intensity)	Relative Intensity after Normalization	Relative to no exposure
untreated	0.053	31.957	1.00	0.05	1.00
4	0.071	33.855	0.94	0.07	1.26
8	0.124	35.899	0.89	0.11	2.08
12	0.222	36.880	0.87	0.19	3.63
16	0.499	36.652	0.87	0.44	8.21
24	0.553	36.960	0.86	0.48	9.02

Figure 15: Time course experiments of Giardia trophozoites exposed to nitrite to determine changes in gFlHb protein expression. **A)** Time course of gFlHb expression in response to 20 mM sodium nitrite with chemiluminescent detection. 40 µg protein lysate was loaded per lane. **B)** Ponceau staining of the blot. **C)** Normalization of gFlHb protein expression to total protein from Ponceau staining. Since the 52 kDa band for gFLHb is not visible in the untreated/no nitrite lane, it is necessary to repeat this experiment to obtain a more accurate calculation of the fold change for this protein.

3.4 Location of gFlHb in Giardia Trophozoites by Immunofluorescent Microscopy

The localization of gFlHb was determined by immunofluorescent microscopy for untreated trophozoites and after 24-hour treatment with either $NaNO_2$ or GSNO. (DETA-NONOate treatment was not done as the Leica epi-fluorescence microscope was broken from March 2015 to January 2016). The anti-gFlHb antibody was found to be specific due to the lack of fluorescence seen after incubation with the pre-immune serum (Figure 16). In contrast to the results from Western blotting, gFlHb could be detected in untreated cells (Figure 16) where it was observed in the cytoplasm, and in an area adjacent and between the nuclei**;** the later location could possibly be the basal bodies or the peripheral vacuoles (PV).

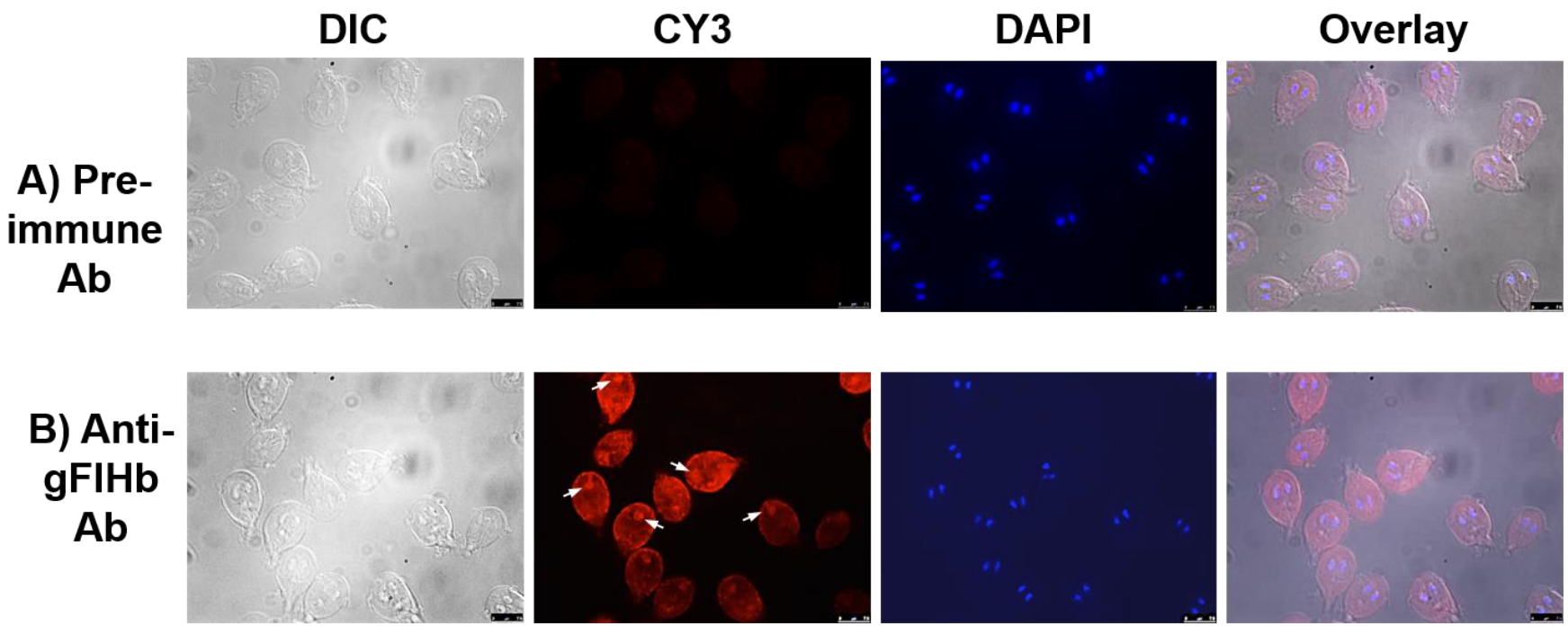

Figure 16: Immunofluorescent microscopy of Giardia trophozoites stained with pre-immune serum (A) and anti-gFlHb antibody (B). **DIC** images of Giardia trophozoites. **DAPI** stains nucleic acids and the nuclei are seen in blue. The slide was incubated with pre-immune serum or anti-gFlHb Ab and then incubated with a **CY3**-tagged secondary antibody (red). The exposure time was 500 ms. **Overlay** of DAPI, CY3 and DIC together. **A)** The pre-immune serum (CY3) was found to have weak cytoplasmic staining which could also be background staining. **B)** The anti-gFlHb antibody showed that gFlHb had cytoplasmic and concentrated staining in an area above the two nuclei in each cell (indicated with white arrows).

Treatment with GSNO or either nitrite caused a variety of morphological changes in the trophozoites, such as changing from a teardrop to a rounded shape, enucleation or enlargement of the nuclei (Figure 17). Moreover fewer cells are detected 24 hours after the addition of these agents, consistent with the cytotoxicity of RNS donors on Giardia trophozoites (Figure 18) (Fernandes and Assreuy 1997). However, there were still trophozoites present which had a similar appearance to the untreated cells, indicating that some cells were able to tolerate nitrosative stress. The location of gFlHb in trophozoites exposed to GSNO or nitrite resembled that of control cells, with possibly more concentrated staining near the cell membrane (Figure 19).

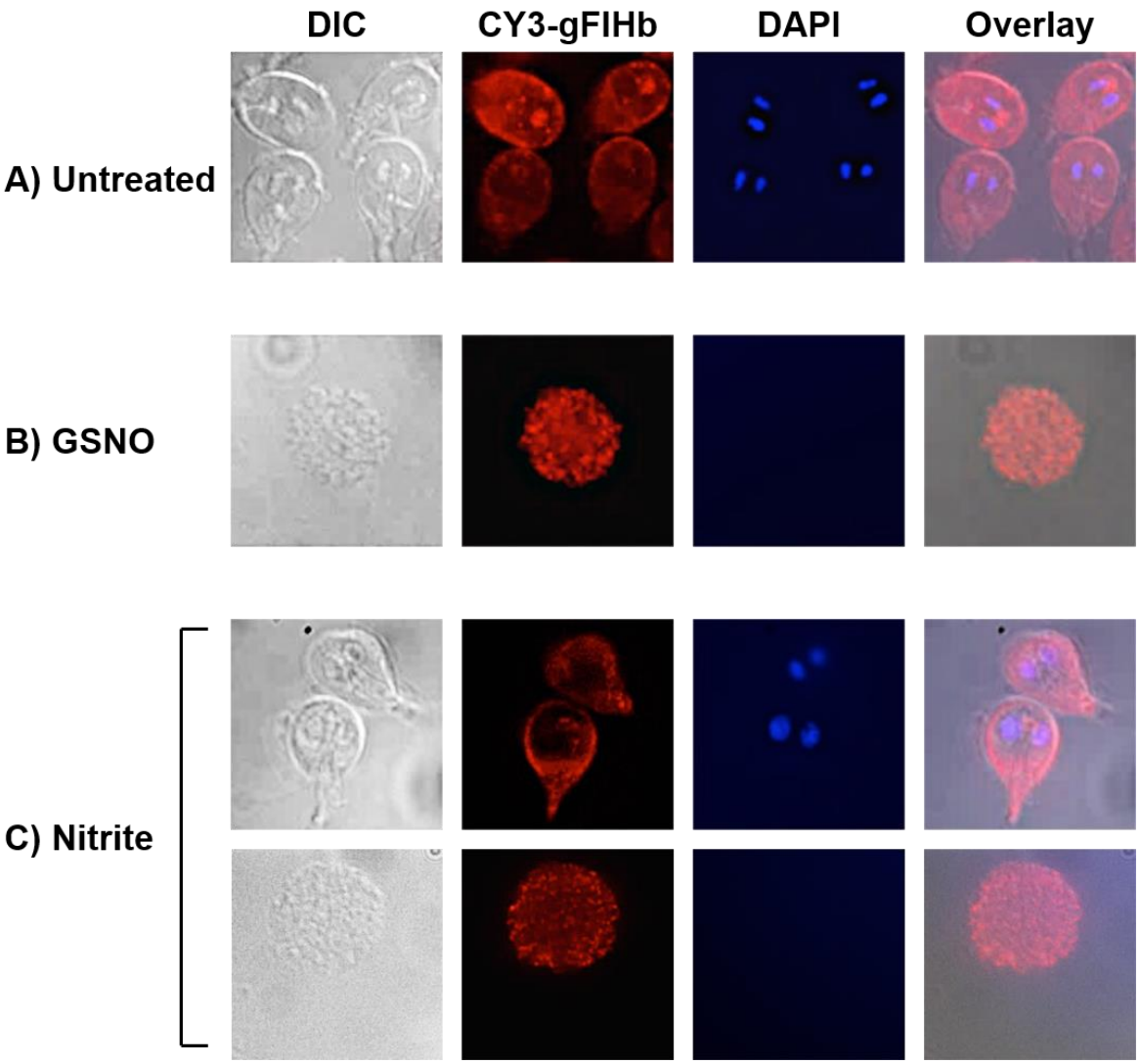

Figure 17: Changes in Giardia trophozoite morphology in untreated cells **(A)** and after 24-hour exposure to either 5 mM GSNO **(B)** or 20 mM nitrite **(C).** Light microscopy images appear in Column 1 (**DIC)**. In the second column **(CY3-gFlHb)**, cells were incubated with anti-gFlHb antibody followed by a CY3-tagged secondary antibody (red). In column 3 (DAPI), the nuclei are labelled in blue. The final column is a overlay of the images in Columns 2 and 3.

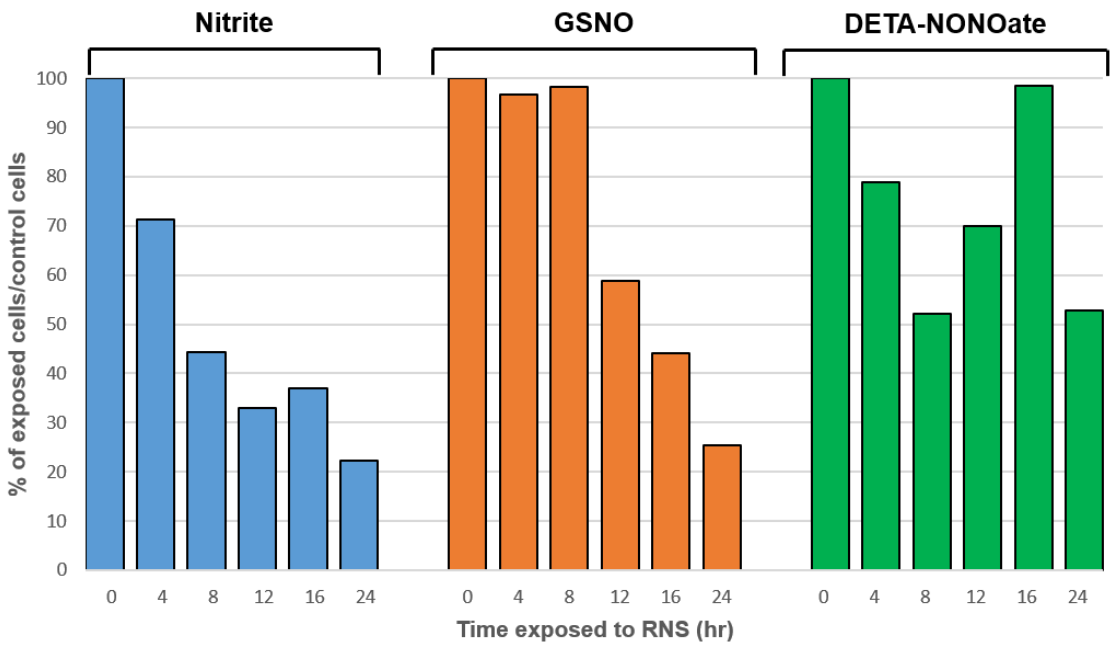

Figure 18: Percentage of the cell survival for different RNS donors over time.

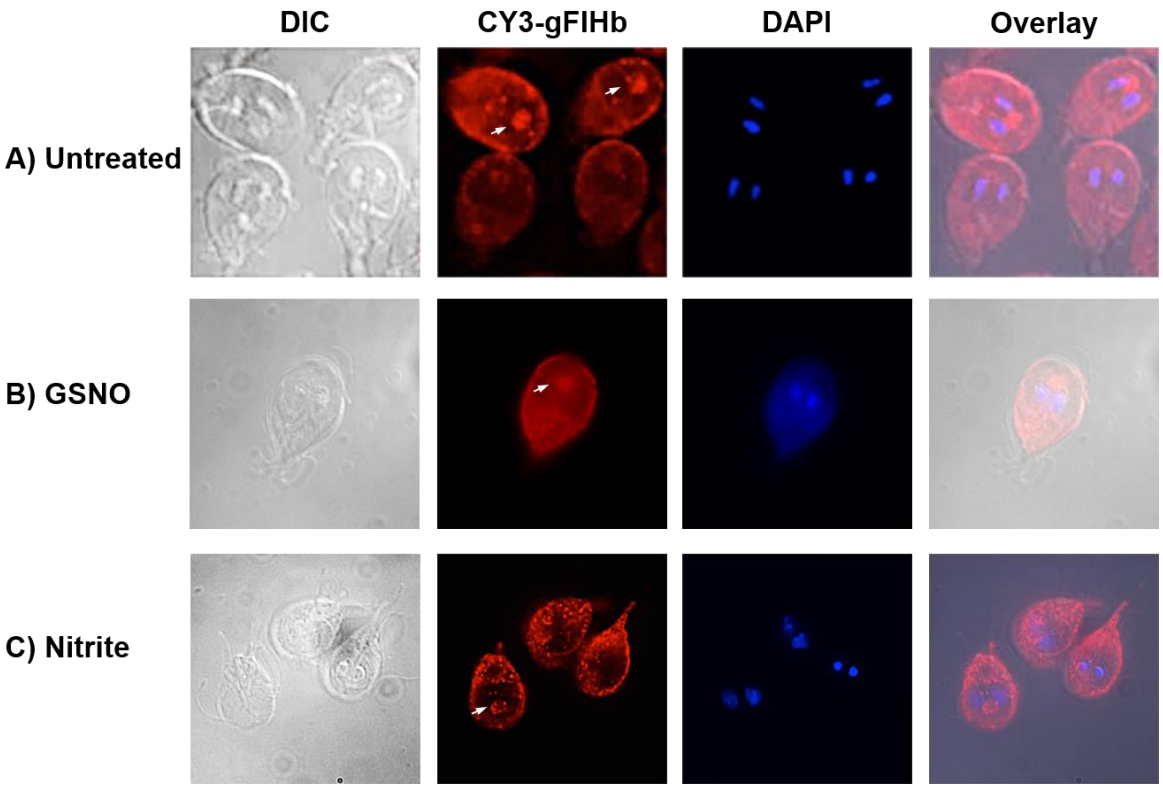

Figure 19: Localization of gFlHb in in untreated trophozoites **(A)** and after 24-hour exposure to either 5 mM GSNO **(B)** or 20 mM nitrite **(C).** Light microscopy images appear in Column 1 (**DIC)**. In the second column **(CY3-gFlHb)**, cells were incubated with anti-gFlHb antibody followed by a CY3-tagged secondary antibody (red). In column 3 (DAPI), the nuclei are labelled in blue. The final column is a overlay of the images in Columns 2 and 3. The white arrows in the CY3-gFlHb images indicate the area of intense staining in the region above the two nuclei.

DISCUSSION

4.1 Context of this work

My results showed that treatment of Giardia trophozoites with RNS donors led to an increase of gFlHb protein expression over a 24-hour period. These observations were supported by RT-qPCR data acquired by Dr. Janet Yee's laboratory. In Dr. Janet Yee's laboratory, they showed that both GSNO and DETA-NONOate exposure increased the gFlHb mRNA levels after 12 −24 hour exposure. This evidence for transcriptional regulation of gFlHb expression is consistent with what is known about flavohemoglobin expression in bacteria and yeast, although the identity of the responsible transcription factors and regulatory sequences are unknown.

Mastronicola and coworkers also exposed Giardia trophozoites for a 24-hour period to the same set of RNS donors under similar conditions, using Western blotting and chromogenic detection to observed changes in gFlHb protein levels (2010). gFlHb protein expression noticeably increased at 10 mM nitrite and higher concentrations, as did cells exposed to 2 mM DETA-NONOate or 5 mM GSNO. While we observed similar increases we also detected a band near 72 kDa which increased on exposure to the RNS donors that has not been reported previously and that is consistent with a post-translational modification of gFlHb. This post-translational modification is likely covalent in nature because it persists under the denaturing conditions of the SDS-PAGE gel. This and the size of the band suggests ubiquitin or ubiquitin-like modification as they form covalent bonds with targeted proteins. A ubiquitin tag is usually used to tag a protein for degradation and is about 9 kDa. This tag binds covalently to the target protein where multiple ubiquitin molecules bind together and the tagged protein will then

undergo degradation by the 26S proteasome complex. Likely gFlHb is not modified by ubiquitin because when ubiquitin binds the protein many molecules of ubiquitin attach which leads to a braided polyubiquitin chain. The polyubiquitin chain would shift the mass much larger than 72 kDa. However, Giardia has been shown to contain small ubiquitin like modifier (SUMO) protein, and sumoylation has been shown to be present and active in Giardia (Ciechanover and Iwai 2004, Vranych, Merino et al. 2012).

Both ubiquitin and ubiquitin-like proteins are known to bind reversibly to the target protein (Gill 2004). Unlike an ubiquitin tag, only a single SUMO protein usually binds to the target protein. Giardia's SUMO is 12 kDa but when attached to the target protein, it has been shown to have an apparent ~20 kDa shift in the size of sumoylated proteins on a SDS-PAGE gel, supporting the results we see in our Western blots (Park-Sarge and Sarge 2009, Vranych, Merino et al. 2012).

SUMO is a protein which is ubiquitously expressed across the eukaryotic kingdom. Like ubiquitinylation, sumoylation results in the formation of an isopeptide bond between the C-terminal glycine residue of the modifier protein and the ε-amino group of a lysine residue in the acceptor protein. This modification is usually in response to an external or internal stimuli such as cell-cycle transition, nutritional state, heat shock, oxidative stress and DNA damage (Geiss-Friedlander and Melchior 2007). Many roles of SUMO are known in the nucleus including regulation of transcription, chromatin structure and DNA repair. SUMO also has a variety of roles in the activation of enzymes and transcriptional regulators, routing of proteins to their sub-cellular destination, promoting specific protein-protein interactions, altering protein stability or activity of the target protein, and even protecting the target protein from ubiquitin-mediated degradation.

It is possible that sumoylation of gFlHb could result in a change in localization, such as seen after nitrite exposure, or to stabilize the protein (Ciechanover and Iwai 2004).

No previous work has been reported on the connection of nitrosative stress and protein sumoylation. But there has been work done on yeast cells exposed to stressors such as hydrogen peroxide or ethanol, in which an increase in protein sumoylation was detected by mass spectrometry (Zhou, Ryan et al. 2004). Two of the identified sumoylated proteins were stress response enzymes: superoxide dismutase, which converts O_2^- to O_2 and hydrogen peroxide; and peroxiredoxin, an antioxidant which can reduce H_2O_2 to water. These data would suggest that flavohemoglobin has the possibility to be sumoylated in response to nitrosative stress in Giardia.

We were provided with an anti-Giardia SUMO monoclonal mouse antibody from the laboratory of Maria Touz, who studies the roles of SUMO in Giardia (Vranych et al, 2012). We had hoped that this antibody would also detect a 72 kDa band on blots of RNS-donor treated trophozoites. However, we tested this antibody on untreated trophozoites and found that we could not detect the SUMO or any bands for sumoylated proteins on the blot. The secondary anti-mouse antibody with the SUMO antibody was tested with a primary antibody against α-tubulin and was found to give the expected size for tubulin bands on a Western blot. This suggest that the lack of detection of SUMO bands on Western blots is unlikely to be due to the secondary antibody. The hybridization stringency of conditions were lowered by incubating the blot overnight with a low dilution (300-fold) of primary antibody, as well as low amounts of Tween-20 (0.01%) in the washes. Even under these conditions no bands were detected, which suggested a

problem with the anti-Giardia SUMO mouse antibody, and no further work was done with it.

Another method to determine if gFlHb is sumoylated is through an immunoprecipitation assay. The anti-gFlHb antibody could be immobilized on suitable inert support such as Dynabeads; the RNS-donor treated Giardia lysate would then be incubated on the beads, allowing flavohemoglobin to bind. Once eluted from the beads the recovered proteins could be analyzed by mass spectrometry to determine whether flavohemoglobin was sumoylated, as this would lead to a predictable increase in the mass of the protein.

4.2 Time course of gFlHb protein expression

Time course experiments were done to determine at which point gFlHb protein expression increased after exposure to the three different RNS donors. In these experiments an increase in gFlHb protein levels was detected sooner (8 hours) in DETA-NONOate and GSNO treated trophozoites compared to nitrite-treated trophozoites (12 hours). This difference is likely due to the different rates of release and concentration of NO from each RNS donor.

DETA-NONOate has the simplest and most predictable decay by first-order kinetics, and directly releases NO. DETA-NONOate can release up to 2 mols of NO per mol of reactant in solution. The rate of NO release from DETA-NONOate is pH-dependent, and it increases with decreasing pH. DETA-NONOate has a half-life of ~21 hours in 37°C at pH 7, therefore the addition of 1 mM DETA-NONOate to the Giardia culture medium, which is buffered to pH 7.4 will likely release ~1 mM of NO at 37°C in 24 hours into the Giardia media (Aga and Hughes 2008).

The second RNS donor, GSNO, reacts mainly through transnitrosation reactions with other thiols. When GSNO undergoes transnitrosating reactions it donates a nitrosonium group (NO^+) to another thiol group. The rate of this reaction depends on the particular thiol, and as a result the second-order rate constants are in the range of 1-300 $M^{-1}sec^{-1}$ at ambient temperature (Broniowska, Diers et al. 2013). On its own GSNO cannot be taken up by the cell but by undergoing transnitrosation reactions GSNO can transfer a nitroso group to L-cysteine resulting in S-nitroso-L-cysteine (L-CysNO) which is easily transported into cells (Broniowska, Diers et al. 2013). Once in the cell, L-CysNO can either S-nitrosate cellular glutathione to reform GSNO inside the cell or directly S-nitrosate protein thiols to elicit the majority of biological effects seen from GSNO exposure (Broniowska, Diers et al. 2013). Another slower reaction of GSNO with thiols is through homolytic decomposition. This decomposition produces reduced glutathione disulphide and many lower nitrogen oxides including nitrous oxide, ammonia, hydroxylamine and sulfonamides. However, this reaction is slow, with a second order rate constant of 0.01 $M^{-1}sec^{-1}$ at 37°C (Broniowska, Diers et al. 2013). To estimate the release of RNS from GSNO in experiments on Giardia trophozoites, Eva Yap of Dr. Yee's lab used the Griess reaction to determine that 2 mM of GSNO in TYI-S-33 media produced about 120 μM of nitrite over 24 hours (2011). If this is scaled up to 5 mM of GSNO it is likely that 300 μM of nitrite should be produced over 24 hours. Nitrite measurement is commonly used to detect RNS, as it is an end product of both NO and S-nitrosothiols. But is complicated as it can also be a source of RNS, depending on the conditions (Yap 2011, Broniowska, Diers et al. 2013).

The last RNS donor that we examined was nitrite. The main mechanism for decomposition from nitrite to NO is under acidic conditions where NO_2^- is protonated to HNO_2 which forms both NO and NO^+ (Ridnour, Thomas et al. 2004, Spiro 2006**)**. While the Giardia culture medium is initially buffered to pH 7.4, this will fall below pH 7 as the cells produce organic acids that are the end products of anaerobic glycolysis and other fermentation pathways. This may explain why it takes more time for gFlHb protein levels to rise when exposed to nitrite as an RNS donor, as it is only when the medium becomes acidic that nitrite will generate nitric oxide. On the other hand, an additional pathway for NO production from nitrite also exists. Nitrite is also known to interact with heme-containing proteins in bovine serum albumin, which act as nitrite reductases and can catalyze the formation of nitric oxide from nitrite (Mikoyan, Kubrina et al. 2006). As calf serum is a component of the culturing medium this could be a mechanism of nitrite decomposition. This pathway, however, is much slower than the generation of NO from nitrite under acidic conditions. There is no easy way to measure how much NO is released by nitrite in our growing conditions, as the most commonly used colorimetric assay to estimate the production of NO, the Griess reaction, actually detects nitrite as the end product. To determine NO directly would require an NO-sensing electrode. Mastronicola and coworkers did not measure the amount of NO produced by nitrite decomposition in the media (2010).

Giardia may encounter nitrosative stress from various sources and at different life cycle stages. There are three main sources of NO production in the lumen of the small intestine, including microflora by-products, host nitric oxide synthase activity and dietary sources. Since the proximal small intestine has fewer microorganisms and nitrogen oxides

than the large intestine, and NO reduction occurs under anaerobic respiration, these are unlikely to be the main source of NO that would affect Giardia. The second source of NO release in the small intestine is from host NOS which includes both eNOS and iNOS. eNOS likely will not produce enough NO to affect Giardia trophozoites' survival as it will be in picomolar concentrations of NO over short periods of time whereas iNOS will continuously produce NO at micromolar levels (Kim, Kim et al. 2002, Kleinert, Pautz et al. 2004). The last form of potential nitrosative stress to trophozoites is through dietary sources. Our saliva contains about two-thirds (60-210 μM) of the nitrite going to the stomach, and the acidic conditions of the stomach convert nitrite to NO (Kelm 1999). As a result, the concentrations of NO in the stomach are known to oscillate between 10-100 ppm or about 0.33-3.3 μM of NO (Lundberg and Weitzberg 2013). Giardia cells excyst in the stomach and therefore may experience nitrosative stress, at least transiently until they reach the small intestine. It is possible that there are residual effects from NO generation in the stomach which would still be present in the small intestine where the trophozoites reside (Forrester and Foster 2012, Lundberg and Weitzberg 2013, Pereira, Ferreira et al. 2013).

The slow response of Giardia trophozoites to RNS is puzzling, especially when compared to other microorganisms. In *Candida albicans,* Ullmann and coworkers noted a rapid increase in flavohemoglobin mRNA expression in response to NO gas or sodium nitrite, with increased expression seen after 5 minutes' exposure to nitrite (2004). Why does it take Giardia take so long to express gFlHb protein if NO is in a high concentration in the stomach and small intestine? I propose that gFlHb is likely needed during excystation or shortly afterwards due to the high levels of RNS and oxygen in the

stomach and small intestinal tract. This would require further experiments on gFlHb expression on Giardia cysts. While trophozoites in culture can be stimulated to encyst, the efficiency of *in vitro* encystation is about 40% and the efficiency of excystation of these is less than 10% (Einarsson, Troell et al. 2016). As trophozoite cultures are not readily scaled up, there is very limited published data available on experiments that involve encystation/excystation *in vitro*.

4.3 Immunofluorescent microscopy

After the exposure of trophozoites to GSNO or nitrite there was a change in localization of gFlHb from the cytoplasm to towards the cell membrane. This could be because NO and O_2 are about 9x more soluble in hydrophobic solvents than in aqueous solution and therefore are concentrated in the hydrophobic regions of the cell (Liu, Miller et al. 1998, Ridnour, Thomas et al. 2004). It is possible that gFlHb is present to protect the membrane from nitrosative damage.

Giardia contains peripheral vacuoles (PV) which also have a similar cellular localization to gFlHb, above the nuclei. These vesicles are present on the dorsal side of Giardia trophozoites under 2-dimensional images and have shown both punctated staining of the outer membrane and staining of an area adjacent to the nuclei. These peripheral vacuoles are connected directly to the periphery of the cell via the plasma membrane and some are connected directly to the endoplasmic reticulum. The likely role of PVs is to undergo fluid phase endocytosis, which allows the sampling of the ever-changing environment surrounding the trophozoites and could possibly gather resources for the endoplasmic reticulum. One of these resources could be heme. It is possible that gFlHb would have similar staining to the PVs if it acquired heme from this organelle, as Giardia

does not synthesize heme. gFlHb may not be present within the PV but likely adjacent to it, as PVs open and close constantly to the surrounding environment, and anything present in the PV would be excreted from the cell. To determine if gFlHb is located near or within the peripheral vacuoles, fluorescently-tagged dextran can be used as a marker for the latter. Within a 20 minute incubation, tagged dextran will be taken up within the PV of Giardia trophozoites but it will not pass the through membrane of the vacuole (Gaechter, Schraner et al. 2008). In this way localization of gFlHb and tagged dextran can be compared by fluorescent microscopy to determine whether gFlHb is within the PV or at the PV surface (Zumthor, Cernikova et al. 2016).

The IFA location of gFlHb to the region adjacent to the nuclei within the Giardia trophozoite is also similar to the location of the basal bodies. There are eight basal bodies in Giardia trophozoites which are found between the two nuclei (McInally and Dawson, 2016). Basal bodies are microtuble organizing centers which nucleate flagella/cillia and are the spindle poles during cell division (Lauwaet et al., 2011). Centrin localizes to the basal bodies and an anti-centrin antibody can be overlaid on the anti-gFlHb antibody staining to determine if gFlHb localizes to the basal bodies (Lauwaet et al., 2011).

Since there was no noticeable change in localization of gFlHb after Giardia was exposed to nitrosative stress, the potential SUMO modification of gFlHb does not appear to cause a change in localization of gFlHb. On the other hand, one of the functions of SUMO is to protect the target protein from ubiquitination. Expression of flavohemoglobin under non-nitrosative stress conditions has been shown to have detrimental effects to the host cell, so the function of SUMO binding to gFlHb under nitrosative conditions may be

to stabilize the protein when its activity is needed (Wu, Corker et al. 2004, Bang, Liu et al. 2006, Gilberthorpe, Lee et al. 2007).

4.4 Conclusion

Giardia flavohemoglobin protein expression increased after exposure to RNS donors after 24 hours. This confirmed the previous results of Mastronicola and coworkers. I was also able to determine the timing for the increase in gFlHb protein expression in response to each RNS donor, which had not been done previously. The localization of gFlHb in Giardia trophozoites, untreated and after adding RNS donors, was determined which also has not been done previously. Further work to determine if gFlHb is sumoylated could be done by using an immunoprecipitation assay with an anti-gFlHb antibody followed by mass spectrometry. In addition, further experiments need to be done under conditions that avoid gross changes to the morphology of trophozoites that we observed on prolonged exposure to high concentrations of RNS donors. This could include using lower concentrations of donors, shorter exposures, and/or donors that have faster release kinetics of nitric oxide. In this way we might be able to detect changes in gFlHb expression and localization in the trophozoites that remain relatively normal. To confirm the localization of gFlHb surrounding the peripheral vacuoles of Giardia trophozoites, fluorescently labelled dextran, which enters and stains the PV, can be overlaid with gFlHb stained cells in future immunofluorescent microscopy experiments. Similarly, the alternative location of gFlHb in the basal bodies of Giardia trophozoites can be confirmed by co-localization experiments with an antibody against centrin and gFlHb.

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APPENDIX

Table 1: Cell counts of non-exposed cells and 5 mM GSNO exposure at same time points

Time point and non-exposure/GSNO exposure	Cell Count per tube	% of exposed cells/non-exposed cells
0 hr non-exposed	29.27 x 10^6	100
4 hr non-exposed	18.22 x 10^6	96.78
4 hr 5 mM GSNO	17.63 x 10^6	
8 hr non-exposed	19.05 x 10^6	98.16
8 hr 5 mM GSNO	18.70 x 10^6	
12 hr non-exposed	23.43 x 10^6	58.89
12 hr 5 mM GSNO	13.80 x 10^6	
16 hr non-exposed	21.70 x 10^6	44.01
16 hr 5 mM GSNO	9.55 x 10^6	
24 hr non-exposed	28.41 x 10^6	25.50
24 hr 5 mM GSNO	7.24 x 10^6	

Table 2: Cell counts of non-exposed cells and 20 mM nitrite exposure at same time points

Time point and non-exposure/ Nitrite exposure	Cell Count per tube	% of exposed cells/non-exposed cells
0 hr non-exposed	27.64 x 10^6	100
4 hr non-exposed	37.35 x 10^6	71.32
4 hr 20 mM nitrite	26.64 x 10^6	
8 hr non-exposed	37.38 x 10^6	44.32
8 hr 20 mM nitrite	16.60 x 10^6	
12 hr non-exposed	33.60 x 10^6	33.03
12 hr 20 mM nitrite	11.10 x 10^6	
16 hr non-exposed	40.84 x 10^6	36.90
16 hr 20 mM nitrite	15.07 x 10^6	
24 hr non-exposed	55.36 x 10^6	22.17
24 hr 20 mM nitrite	12.28 x 10^6	

Table 3: Cell counts of non-exposed cells and 1 mM DETA-NONOate at same time points

Time point and non-exposed/DETA-NONOate exposure	Cell Count per tube	% of exposed cells/non-exposed cells
0 hr non-exposed	10.63 x 10^6	100
4 hr non-exposed	23.49 x 10^6	78.92
4 hr 1 mM DETA-NONOate	18.53 x 10^6	
8 hr non-exposed	23.68 x 10^6	52.18
8 hr 1 mM DETA-NONOate	12.36 x 10^6	
12 hr non-exposed	29.11 x 10^6	69.96
12 hr 1 mM DETA-NONOate	20.37 x 10^6	
16 hr non-exposed	26.40 x 10^6	98.47
16 hr 1 mM DETA-NONOate	26.00 x 10^6	
24 hr non-exposed	31.41 x 10^6	52.91
24 hr 1 mM DETA-NONOate	16.62 x 10^6	