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Lipid Uptake and Metabolism in the Parasitic Protozoan Giardia lamblia.

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LIPID UPTAKE AND METABOLISM IN THE PARASITIC PROTOZOAN *GIARDIA LAMBLIA*

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Dedication

This dissertation is dedicated the friends, family and colleagues who have supported and encouraged me through the last five years, but especially to Añin and Aye.

LIPID UPTAKE AND METABOLISM IN THE PARASITIC PROTOZOAN *GIARDIA LAMBLIA*

by

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DISSERTATION

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Abstract

Giardia lamblia is a protozoan parasite that causes various intestinal syndromes, and it is a common cause of water-borne illness worldwide, both in developed and developing countries. *Giardia* attaches to the mucosal epithelia of the duodenum below the bile duct, where it is exposed to bile salts and dietary lipids. *G. lamblia* is unable to synthesize lipids *de novo* and must therefore scavenge necessary lipids from its extracellular environment and remodel them as needed. However, the current lipidomic analysis (presented in this dissertation) has revealed that while the *Giardia* lipidome is rich in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG), its growth medium contains only PC, lyso-PC, and some diacylglycerol. Therefore, it is quite likely that *Giardia* has more lipid synthesis abilities than previously reported. The lipid analysis also includes fatty-acid analysis by GC-MS, which has revealed that *Giardia* trophozoites, encysting cells, and *in vitro*-derived cysts contain odd carbon-chain fatty acids (OCFAs), as well as a number of fatty acids not present in the medium, indicating that elongases and desaturases are also active in *Giardia*.

The *Giardia* Genome Database (giardiaDB.org) was searched for the presence of genes encoding enzymes for the synthesis of PG and PE—i.e., two newly synthesized lipids in *Giardia*. Analyses identified phosphatidylglycerol phosphate synthase (PGPS) and phosphatidylserine decarboxylase (PSD) genes, which are expressed throughout the *Giardia* life cycle. Furthermore, I also searched for the genes linked to the synthesis of phospholipid transporters that could be involved in importing phospholipids from outside sources. Interestingly, the search indicated that *Giardia* has the genes for flippase enzymes, which indicates that this parasite relies on scavenging lipids from the host. In order to further elucidate the mechanisms of lipid uptake and incorporation, I labeled cells with [^{13}C]-glycerol isotope to determine whether generation of PG

occurs via the base- or head-group exchange reactions between PC and glycerol. Side by side, I have also used fluorescent lipids as reporter molecules to understand whether *Giardia* uses flippase enzymes to uptake lipid molecules. These studies indicate that PG is indeed generated via head-group exchange and that uptake of PC and PE is completely dependent upon internalization through a flippase-like transporter. The current investigation suggests that *Giardia* has some capability to synthesize its own phospholipids *de novo* but that it mostly depends upon the supplies from outside sources. I speculate that lipid synthesis and transport systems that are operative in *Giardia* are interesting and may serve as potential targets for developing new therapies against this waterborne pathogen that affects millions of children worldwide, especially in poor countries.

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Chapter 1: Introduction

Giardiasis is a common disease worldwide and occurs in humans as well as in livestock, cats and dogs. In developed countries, it is common amongst day-care aged children as well as backpackers and campers. In developing countries, it is widespread primarily because of the association of the disease with contaminated water supply (CDC 2004) and the World Health Organization estimates that approximately 200 million people are infected each year. Giardiasis manifests itself as various intestinal syndromes, including diarrhea, malabsorption, and stomach cramps (Adam 2001). While the infection clears without treatment in the majority of patients, it can persist in immunocompromised patients, particularly those deficient in IgA (Langford et al. 2002). In the United States, giardiasis is considered a public health risk and has also become a concern of food industry, because several cases of food-borne giardiasis (with seven outbreaks) were reported since 1993 (Rose and Slifko 1999). *Giardia* is also considered a class B biodefense agent (Thompson 2000).

1.1. Giardia lamblia *as a human pathogen*

The trophozoite form of *Giardia lamblia* colonizes the lumenal surface of the human small intestine, is non-invasive and multiplies by asexual reproduction (binary fission). Increased giardiasis can be attributed to dense populations, poor sanitation, and lack of clean drinking water and/or environmental pollution. Infection begins with ingestion of water-resistant cysts. It is estimated that only ten cysts or less are needed to contract the infection (Talal and Murray 1994). Once ingested, cysts travel through the gastrointestinal tract to the stomach, where the stomach acid facilitates the process of excystation through an unknown mechanism. Newly

excysted trophozoites travel further downstream in the small intestine and colonize below the bile duct and causes giardiasis. Trophozoites can also travel to the jejunum, where they encyst (Figure 1). During their colonization in the small intestine newly-generated cysts are passed in the feces, thus continuing the cycle. (Adam 2001). While genes coding for toxins have not been found in the *Giardia* genome (Morrison et al. 2007), and the cause of symptoms is not well characterized, though it is thought that the malabsorption and diarrhea are caused by epithelial barrier dysfunction, as a result of epithelial apoptosis as well as decreased expression of the tight junction proteins claudin-1, -2, -4, and -7, and occludin (Troeger et al. 2007).

The small intestinal environment is extremely harsh for trophozoites. In a series of pioneering experiments, Gillin et al. (1988) have demonstrated the process of encystation, by injecting trophozoites into the stomachs of mice, following the fate of trophozoites and understanding the process of encystation. Interestingly, the largest numbers of trophozoites and cysts were found in the jejunum, and fatty acids and bile salts with slightly alkaline pH (7.8) were found to be stimulators of encystation (Lauwaet et al. 2007). Lujan et al. (1996) subsequently showed that starvation for cholesterol was necessary and sufficient to induce encystation. More recently, Hernandez et al. (2008) demonstrated that sphingolipid biosynthesis is also important for giardial encystation

Giardiasis is commonly treated with nitroheterocyclic compounds such as metronidazole and tinidazole (Figure 2). However, resistance by the parasite to these chemotherapeutics has been reported, and patients tend to suffer severe side-effects to the drugs (Upcroft and Upcroft 2001). The severe side-effects of metronidazole are likely due to the mechanism of action of the drug. Metronidazole targets the glycolytic pathway—more specifically, it is reduced by pyruvate ferredoxin-oxidoreductase (PFOR) into its active form (Harris et al. 2001). The action of PFOR

ultimately leads to the inhibition of DNA segregation and cell cycle arrest. PFOR is only found in anaerobic organisms (such as anaerobic protozoans such as *Giardia*, *Entamoeba*, and *Trichomonas*, as well as anaerobic bacteria), and replaces the function of the pyruvate dehydrogenase found in aerobic organisms (Upcroft and Upcroft 2001). This provides some degree of specificity, since the drug itself is relatively non-toxic until it is reduced by PFOR (Harris et al. 2001). However, because PFOR is also found in bacteria, metronidazole is considered a wide-spectrum drug. It is likely that metronidazole is cytotoxic to the bacterial flora of the intestinal tract, therefore causing the increase of diarrhea common to this treatment.

1.2. The giardial genome

The giardial genome is distributed through five chromosomes and is approximately 11Mbp is length and 6470 open reading frames (ORFs) have been identified. Of the identified ORFs, a number of them are similar to bacteria or archaea (Morrison et al. 2007). This can likely be attributed to lateral gene transfer. *Giardia*, like *Entamoeba* and *Trichomonas*; is exposed to bacteria throughout its life cycle, including during infection, giving all three protozoan parasites ample opportunity to exchange genes with bacterial cells . Other intestinal protozoans also have similar genome sizes—*Cryptosporidium parvum*, for instance, has a 9Mbp genome spread across eight chromosomes, but with only 3807 genes, and like *Giardia*, has very few introns (only 5 percent of genes have introns) (Abrahamsen et al. 2004).

Giardia is a binucleate protozoan, belonging to the family of *Diplomonads*, and each nuclei has two complete copies of the genome in a stationary phase trophozoites (Yu et al. 2002; Keeling 2007). However, it has been proposed that the number of gene copies varies anywhere

from 4N to 16N throughout the life cycle, which trophozoites ranging from 4N to 8N, depending on the cell cycle. Cysts, on the other hand, have 4 nuclei, giving rise to gene copy numbers ranging from 8N to 16N (Bernander et al. 2001). The binucleated nature of this protozoan, as well as its high gene copy number, is therefore a limitation in generating gene knockdowns and other transfections.

1.3. Molecular mechanisms of giardial differentiation

Giardia is an evolutionarily basal eukaryote, having branched off from prokaryotes early on. However, because of its unique position in the evolutionary tree, it shares similarities with both eukaryotes and prokaryotes (Figure 3). Like other eukaryotes, it has distinct nuclei and enclosed organelles, such as an endoplasmic reticulum. However, *Giardia* does lack some organelles, such as distinct mitochondria, peroxisomes, and has only a transient Golgi (Adam 2001). This transient Golgi appears only during encystation (also called encystation secretory vesicles, or ESVs) (Reiner et al. 1990; Gillin et al. 1991; Lanfredi-Rangel et al. 1999) and is responsible for the transport of cyst wall proteins to the cell membrane (Gillin et al. 1991). While *Giardia* lacks a mitochondrion, it has been proposed that this protozoon does possess a mitosome; which is thought to be a vestigial mitochondrion. It is possible that *Giardia* acquired a mitochondrion through and endosymbiotic event, but later its function was lost (Tovar et al. 2003; Regoes et al. 2005). The lack of peroxisomes is logical, given this parasite's limited lipid metabolic abilities

Differentiation into cysts begins with the internalization of flagella as well as breakdown of the ventral disk, causing trophozoites to detach (Lauwaet et al. 2007). In addition, synthesis of

cyst wall proteins (CWP) 1, 2, and 3 begins, and these acidic proteins are transported to the plasma membrane by ESVs (Reiner et al. 1990; Gillin et al. 1991). Once CWPs are deposited and the cyst wall is formed, the cell is no longer motile and is rounded, rather than pear-shaped (Adam 2001; Lauwaet et al. 2007). During encystation, a trophozoites in G1 phase also undergoes two complete DNA replication cycles, without subsequent cytokinesis, resulting in a cyst with four nuclei and a total gene ploidy of 16N. Thus, a cyst is composed of two trophozoites (Bernander et al. 2001). The cyst wall is composed of 40% protein, and the remainder is composed of carbohydrates and lipids, with the primary carbohydrate being N-acetylgalactosamine (Jarroll et al. 1989; Gerwig et al. 2002). This tough exterior acts to protect the cyst from the environmental conditions present outside the host, including temperature changes and water.

While the exact mechanisms that trigger encystation and excystation are not well defined, it has been previously suggested that exposing cells to conditions that most closely resemble those within the host are most effective at producing in vitro-derived trophozoites from excystation (Bingham et al. 1979). In the host, cysts are exposed to highly acidic conditions as they pass through the stomach, followed by a quick neutralization once in the duodenum. In the small intestine, because of the site of colonization below the bile duct, trophozoites are exposed to bile salts and digestive enzymes with detergent activity. In the jejunum, where excystation likely occurs, *Giardia* is exposed to lactic acid, a by-product of the metabolic activities of the bacteria present. Furthermore, it has been shown that exposure to low pH and pancreatic proteases is crucial in the excystation process (Boucher and Gillin 1990). An excyzoite (ie: a cyst in the process of excystation) divides twice to produce four trophozoites (Bernander et al. 2001).

1.4. Energy production by Giardia

The conversion of glucose to pyruvate is the major source of carbohydrate-derived energy production in *Giardia* and occurs via the Embden-Meyerhof-Parnas and hexose monophosphate shunt pathways (Adam 2001). Carbohydrate metabolism in *Giardia* is not compartmentalized and occurs in the cytosol (Lindmark 1980), unlike trypanosomatids, which carry out glycolysis in the glycosome (Opperdoes and Borst 1977), or *Trichomonas vaginalis*, in which glycolytic enzymes are found in the cytosol, but the oxidation of pyruvate occurs in the hydrogenosome (Johnson et al. 1993). Glucose metabolism in *Giardia* produces a net two ATPs and one NADH (Adam 2001).

Aside from carbohydrate metabolism, energy is also produced from amino acid metabolism. In particular, alanine, arpartate, and arginine (Mendis et al. 1992; Schofield et al. 1992; Schofield et al. 1995). In particular, the conversion of arginine to ornithine is a favorable reaction for ATP production, because ATP is produced through substrate level phosphorylation, and therefore, oxygen and redox systems are not necessary. Because *Giardia* is microaerophilic, this makes it a favorable reaction. Arginine is rapidly consumed from the media, and the ATP production from the catabolism of arginine to citrulline is 7-8 higher than from glucose (Schofield et al. 1992). This reaction is catalyzed by arginine deiminase, which has also has a role in regulating antigenic variation and has been shown to bind to the 5-amino acid conserved anchor (CRGKA) of variant surface proteins (VSP) (Touz et al. 2008).

1.5. Lipid metabolism in Giardia

Giardia is unable to synthesize lipids *de novo*, and is therefore is dependent on exogenous lipids in order maintain and synthesize membranes and generate lipid-based signaling molecules (Das et al. 2002). *Giardia* colonizes the duodenum below the bile duct, where it is exposed to dietary lipids and bile salts from its host (Adam 2001). These bile salts are thought to be involved in carrier-mediated uptake of lipids (Das et al. 1997). Transport systems for lipid uptake depend largely on their structure. For instance, ceramide, a sphingolipid, is internalized by clathrin-coated vesicles (Hernandez et al. 2007), while uptake of phospholipids in *Giardia* is likely carried out by flippases. Flippases belong to a family of phospholipid transporters, which also include floppases and scramblases. Flippases transport phospholipids into the cell, while floppases are responsible for transporting phospholipids outward, and scramblases are capable of carrying out transport in both directions (Daleke and Lyles 2000). While the mechanism of flippase action has not been previously delineated in *Giardia*, the *Giardia* genome database contains sequences for four flippases, annotated in the database as phospholipid-transporting ATPases (Morrison et al. 2007).

Once transported into the cell, phospholipid (PL) remodeling can occur by either acyl or head-group exchange. Fatty acyl groups are likely cleaved from the PL by a lysophospholipase and exchanged for another fatty acid by a lysophosphatidic acid acyl transferase (Chapoy 2005). It has been shown that trophozoites exposed to [^{3}H]-oleate, -myristate, -palmitate, and –arachidonate are able to incorporate these fatty acids into PC, PG, and PE (Gibson et al. 1999). Exchange of the phosphate head-group likely occurs through the activities of various enzymes. For instance, trophozoites labeled with [^{3}H]-*myo*-inositol incorporate this base into PI as well as

lyso-PI (Subramanian et al. 2000). Also, unpublished data from Das et al shows that when trophozoites are exposed to [^{14}C]-labeled head-groups, these bases are incorporated into phospholipids. In particular, choline incorporates into PC and *lyso*-PC, ethanolamine into *lyso*-PE, glycerol into PG, and interestingly, serine incorporates into PE, rather than phosphatidylserine (PS) (Das 2005). The incorporation of serine into PE indicates that a phosphatidylserine decarboxylase (PSD) is highly active in *Giardia*.

The mechanisms of phospholipid head-group remodeling enzymes differ greatly in *Giardia* from higher eukaryotes, including mammals and even other protozoans. For instance, in *Plasmodium falciparum*-infected erythrocytes, PG, phosphatidic acid (PA), PS, and PI are all directly formed from CDP-DAG (Vial et al. 2003) (Figure 5). Specifically, PG biosynthesis occurs by the conversion of CDP-DAG and glycerol-3-phosphate to phosphatidylglycerolphosphate (PGP) by a PGP synthase. PGP is then dephosphorylated to form phosphatidylglycerol (PG). Two molecules of PG can then be fused to form its dimer, cardiolipin (Xu et al. 1999). PC and PE are generated via the Kennedy pathway, in which ethanolamine and choline are phosphorylated and combined with cytidine triphosphate (CTP) and then DAG to form PE and PC, respectively. These two PLs can also be generated from PS via base-exchange reactions (Vial et al. 2003), which likely involves decarboxylation to form PE, and decarboxylation and methylation to form PC. Infected erythrocytes and Plasmodium have very similar PL pathways—the only difference being that *Plasmodium* is not capable of exchanging the serine base of PS for ethanolamine or choline head-groups (Figure 5) (Vial et al. 2003).

In *Saccharomyces cerevisiae*, both the Kennedy and base-exchange pathways for PE biosynthesis are present, though each is catalyzed by a different enzyme. In this yeast, two isoforms of PSD are present. PSD1 is responsible for the decarboxylation of PS, a reaction which

takes place in the inner mitochondrial membrane. PSD2, localized in the Golgi, is responsible for generation of PE via the Kennedy pathway. PE can also be methylated by PE methyltransferases to form PC (Birner et al. 2001). However, such *de novo* synthesis pathways are thought to be unlikely to occur in *Giardia*, given its evolutionarily basal position. Interestingly, *S. cerevisiae psd1* and *psd2* mutants can be rescued with the addition of choline to the media, which suggests that the presence of PC is essential for the formation of PE (Birner et al. 2001). Similarly, in *Giardia,* trophozoites cultured without serum (the source of PC) have severely reduced attachment (23%) and growth (52%), indicating that PC is a required PL (Lujan et al. 1994).

1.7. The goal of my thesis

The goal of this work is to answer the following questions: (1) Does *Giardia* have the ability to synthesize new lipids, given the previous reports that the lipid profile of this parasite quite likely resembles that of its growth medium? (2) Is it possible for *Giardia* to generate necessary membrane lipids by altering or remodeling phospholipids that have been scavenged from the small intestinal milieu? Because the enzymes and pathways of remodeling reactions are unique, efforts should be taken to determine if they could be used as targets for the development of new therapies against this pathogen.

Giardia is dependent upon exogenous lipids in order to maintain membrane integrity and generate signaling molecules such as phosphatidylinositol-3–kinase (Hernandez et al. 2007), which indicates that such mechanisms could be exploited as targets for developing chemotherapeutic compounds against giardiasis.

In order to be able to obtain the necessary lipids, *Giardia* must have a lipid transport system because facilitated diffusion is likely to be a rather slow reaction. The *Giardia* Genome

database (www.giardiadb.org/giardiadb) shows that sequences encoding four such proteins are present—four copies of a flippase, or a phospholipid ATPase transporter (Morrison et al. 2007). Once transported into the cell via flippases, phospholipids can either be incorporated into membranes "as is" or remodeled via base- or acyl-exchange reactions (Das et al. 2001). In an acyl-exchange reaction, a fatty acid chain is cleaved from the glycerol backbone and replaced with another chain, of either shorter or longer carbon length. Restructuring phospholipids via base-exchange involves replacing the phosphate head group of one phospholipid for another—for instance, replacing the trimethyl amine head group of a PC for a glycerol group to form PG via the action of a phosphatidylglycerolphosphate synthase (PGPS), or decarboxylating a PS to form PE in a reaction catalyzed by a phosphatidylserine decarboxylase (PSD). Both of these enzymes appear to be housekeeping genes because their transcript levels to not vary greatly throughout the life cycle, indicating that maintenance of lipid composition occurs in both trophozoites and cysts.

In order to determine how lipids are metabolized, it was necessary to identify the lipid composition of the cell using mass spectrometry, a technique with high resolving power that was not available when the lipid composition of *Giardia* was previously described. Here, I show that the lipid composition of *Giardia* is much more diverse than that of its growth medium, which indicates that remodeling enzymes are highly active and crucial to the survival of the cell and that *Giardia* is much more capable of generating new phospholipids than previously thought. Furthermore, I have identified a flippase for the transport of phospholipids, as well as remodeling enzymes responsible for the production of new phospholipids. I propose that lipid metabolic pathways in *Giardia* are unique and thus should be considered as new targets for developing potential therapies against *Giardia* and related intestinal parasites.

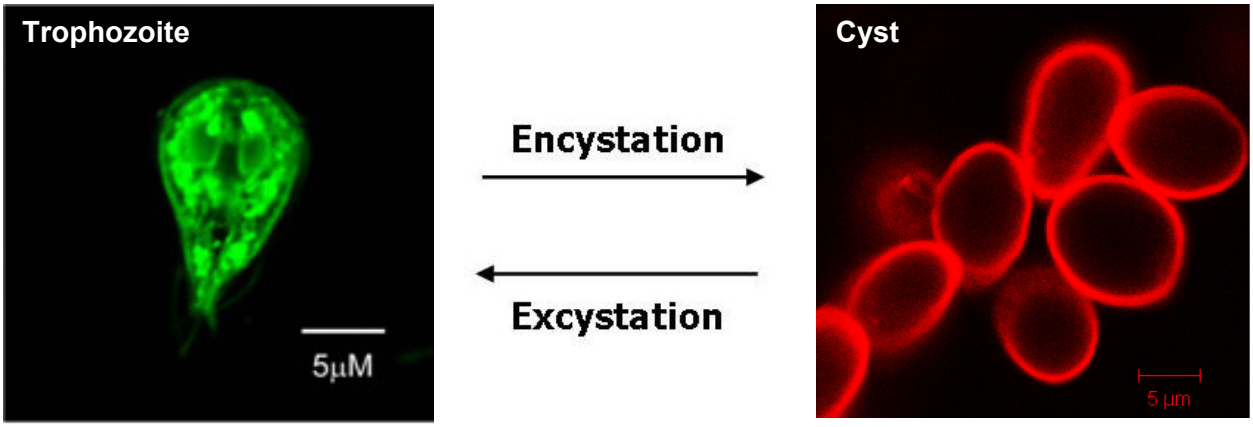
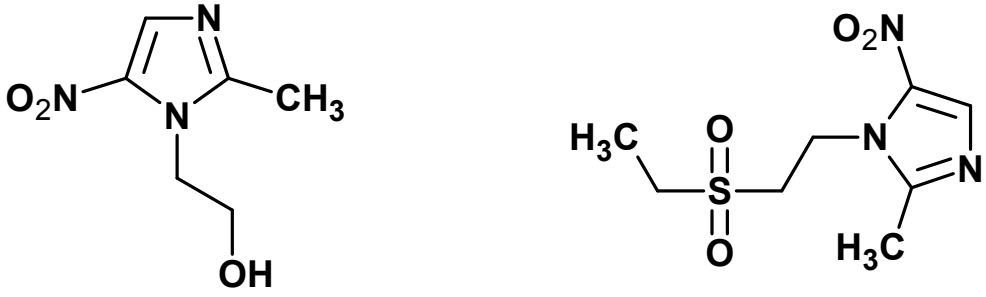


Figure 1. The life cycle of *Giardia lamblia*. Adapted from Hernandez et al. (2007).



Metronidazole **Tinidazole**

Figure 2. Structures of nitroheterocyclic drugs for treatment of Giardiasis.

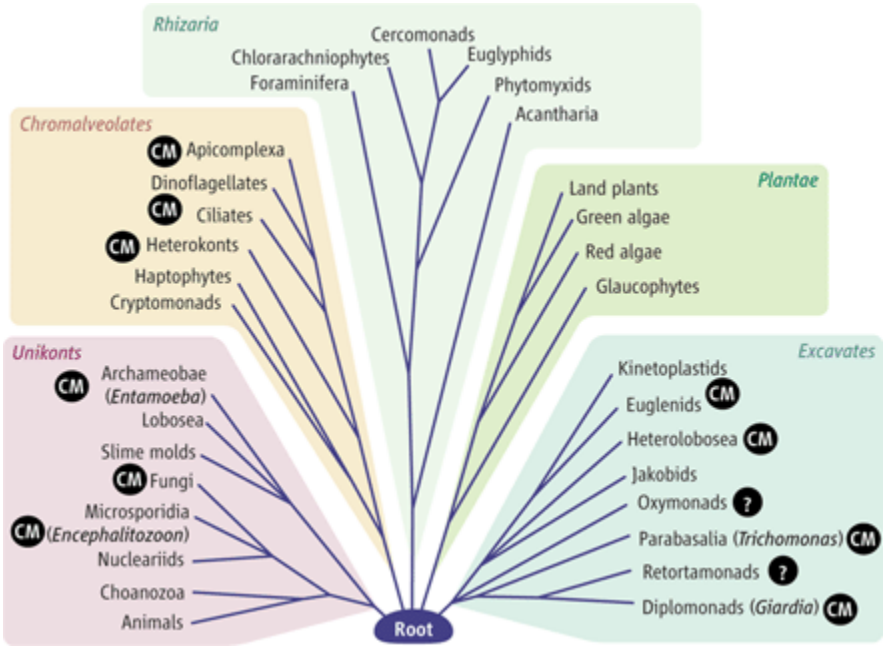
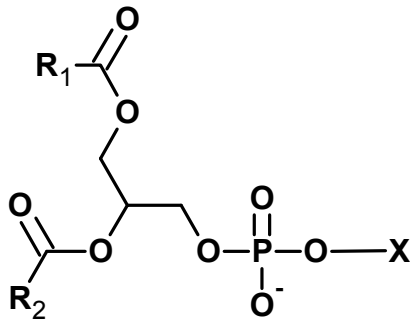
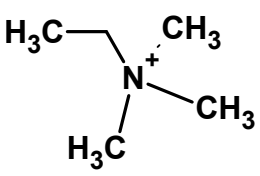


Figure 3. Evolutionary tree showing the evolutionary basal position of *Giardia lamblia* (Keeling 2007).

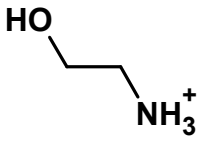
A.



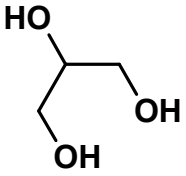
B.



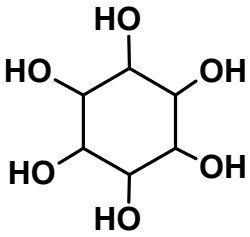
Choline



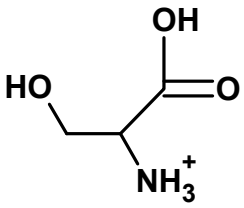
Ethanolamine



Glycerol



Inositol



Serine

Figure 4. Phospholipid structures. A. Structure of a phospholipid, where "X" is a head-group. B. PL head-groups.

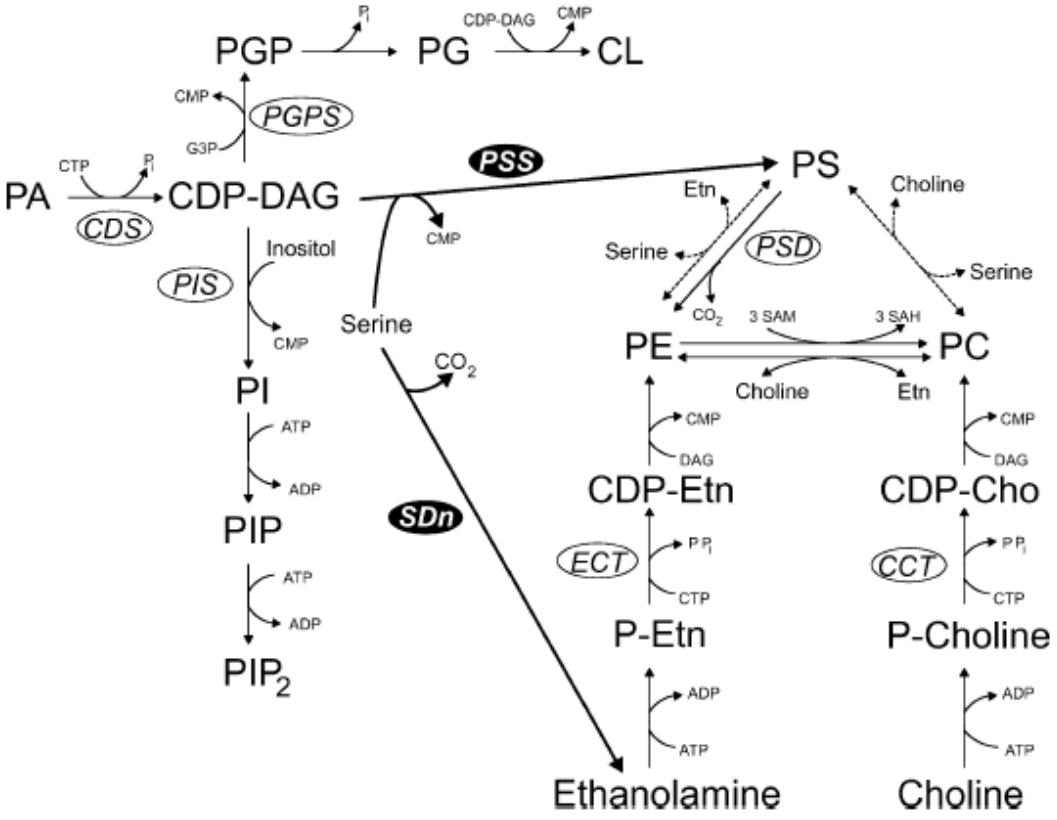


Figure 5. Phospholipid biosynthesis in *Plasmodium* infected erythrocytes.CDS: CDP-DAG synthase, PIP: phosphatidylinositol phosphate, PIP2: PI bis-phosphate, SDn: Serine decarboxylase, PSS: phosphatidylserine synthase (Vial et al. 2003).

Chapter 2: Lipidomic analysis reveals that *Giardia* has the ability to synthesize new phospholipids and fatty acids

The focus of this chapter is to determine the lipid composition of *Giardia* trophozoites, encysting cells, and cysts. Two fundamental questions were asked: (1) Which lipids are present in the growth medium? (2) Does *Giardia* have the ability to synthesize new lipids via *de novo* pathways, given that previous reports have indicated that the lipid profile of this parasite quite likely resembles that of its growth and encystation medium (Jarroll et al. 1981; Kaneda and Goutsu 1988; Mohareb et al. 1991; Ellis et al. 1996), (which contains bovine bile and serum)? Interestingly, those studies were performed using instruments such as high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). While useful, both HPLC and TLC have limited lipid resolution capacities, especially in identifying *Sn*1 and *Sn*2 fatty acids of phospholipids, as well as phosphorylated head groups. Using newly acquired Electrospray Ionization-Quadrupole Time-of-Flight-Mass Spectrometry (ES-QTOF-MS; Micromass Qtof-1, Waters) equipment, it was possible to overcome these challenges and obtain a better resolution. Furthermore, with small amounts of cells (~1 x 10^6), it was possible to quantify the lipid contents using internal standards.

2.1. Materials and Methods

2.1.1 Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. *Serratia marcescens* chitinase and α-N-acetyl-galactosaminidase were purchased

from Sigma-Aldrich and Genway Biotech, Inc. (San Diego, CA), respectively. Phospholipid and fatty acid standards were obtained from Avanti Polar Lipids (Alabaster, AL) and Supelco (Bellefonte, PA), respectively.

2.1.2. Organisms

Giardia lamblia trophozoites (strain WB, ATCC No. 30957) were cultivated in TYI-S-33 medium supplemented with adult bovine serum and bile (Diamond et al. 1978; Keister 1983). The antibiotic piperacillin (100 μg/ml) was added during routine culture of the parasite (Gillin et al. 1989), and parasites were detached by chilling in ice and harvested by centrifugation at 1,500 x g for 10 min at 4 °C, followed by repeated washes. Encystation in culture was carried out by the method of Gillin et al. (1989) by culturing trophozoites in TYI-S-33 medium (pH 7.8), supplemented with bovine serum (10%, v/v), lactic acid (5 mM), and porcine bile (250 mg/ml) for various time points, as described in the text and figure legends. *In vitro*-derived cysts were generated by culturing trophozoites in high-bile medium (TYI-S-33 medium, supplemented with 10% bovine serum and bile, pH 7.8) as described previously by Kane et al. (1991).

2.1.3. Digestion of cysts

In vitro-derived giardial cysts and 48 hour (h) encysting cells were suspended in 0.1 M phosphate buffer (pH 7.0) and incubated overnight at 37 ^{o}C with chitinase (1.5 mg/ml) and α-N-acetyl-galactosaminidase (5.3 Units/ml). Digested cell extracts were subjected to lipid extraction as described below.

2.1.4. Lipid extraction

Non-encysting and encysting *Giardia* trophozoites were grown to confluency and harvested as described above (section *2.2*). Lipids from giardial cell pellets (vegetative and encysting trophozoites), cysts, bovine serum, and bile were extracted 3 times in 10 volumes of each of the following chloroform ($CHCl_3$)/methanol (CH_3OH) solutions: $CHCl_3$:CH_3OH:H_2O (1:2:0.8, v/v/v) and $CHCl_3$:CH_3OH (2:1, v/v). After adding each solvent solution, the tube was vortexed for approximately 1 min, then centrifuged at room temperature for 30 min at 2,000 x g. Following centrifugation, the organic phase was transferred to a clean glass tube with Teflon/PTFE inner disks and stored at -20 °C until further use. The sample was dried under highly pure nitrogen stream after the last extraction in each step (Almeida et al. 2000).

2.1.5. Phospholipid and sterol purification

Glass columns prepared in Pasteur pipettes were packed with fine glass wool and approximately 100 mg silica gel resin (pore size 60 Å, 200–400 μm mesh, Sigma-Aldrich). Columns were washed with CH_3OH and acetone and equilibrated with $CHCl_3$. Following the equilibration, the lipid sample dissolved in 1 ml $CHCl_3$ was loaded onto the column. Neutral lipids (e.g., sterols, triglycerides etc.) and free fatty acids were eluted out with 2–3 ml $CHCl_3$. This was followed by 2–3 ml acetone to elute glycolipids and ceramides, and 2–3 ml methanol to elute phospholipids (Pernet et al. 2006). All samples were dried under nitrogen stream and stored at -20 °C until further use.

2.1.6. Phospholipid analysis by ESI-QTOF-MS

MS spectra for fractionated lipids were acquired in an ESI-QTOF-MS (Micromass Qtof1, Waters). Samples analyzed in positive-ion mode were dissolved in $CHCl_3$:CH_3OH (1:1, v/v) containing 10 mM LiOH. For analysis in negative-ion mode, samples were dissolved in $CHCl_3$: CH_3OH: formic acid: NH_4OH (1:1:0.1%:0.1%). Samples were injected by infusion at a flow rate of 0.5 µl/min, and the capillary voltage was set at 2.5 kV. Full-scan spectra were collected in the 200–2,000 *m/z* range, and MS/MS spectra were automatically collected for each parent ion with abundance higher than 20 counts using ramp-collision energy (20–65 eV) according to the mass range. Argon was used as the collision gas. For quantitative analysis, samples were normalized to 5000 cells/µl and spiked with an internal standard at a final concentration of 2.5 µM C11:0/C11:0-PC for positive-ion mode, or 5 µM C12:0/C12:0-PE for negative-ion mode. Peak height of the standards was normalized to 30% of the total peak height.

2.1.7. Gas chromatography-mass spectrometry (GC-MS) analysis

The analysis of the sterol fraction by GC-MS was carried out as described by Fridberg et al. (2008). For analysis of fatty acids by GC-MS, total lipids were isolated as described above. Alkaline hydrolysis of total fatty acids was carried out following a method adapted from Maldonado et al. (2006). Twenty-five-µl aliquots of the total lipid extracts were dried under nitrogen stream and re-suspended in 100 µl 13 N ammonium hydroxide: methanol (1:1, v:v), then incubated for 1 h at 37 °C and dried under nitrogen stream. The samples were then washed twice with 100 µl of dry methanol, with complete drying under nitrogen stream between each

wash. For the methylation, 100 µl 0.5 N methanolic HCl (Supelco, Sigma-Aldrich) were added, and the reaction mixture was incubated for 1 h at 75 °C. The reaction mixture was allowed to cool to room temperature and then neutralized with 100 µl 0.5 N NaOH. To remove HCl from the reaction, samples were washed with 1 ml each deionized water and dichloromethane (DCM). The aqueous phase was extracted and samples were washed two more times with water. Finally, the organic phase was transferred to a fresh tube and briefly dried under nitrogen stream (Maldonado et al. 2006).

For GC-MS analysis, samples were redisolved in 100 µl DCM and 1 µl was used for analysis in a trace gas chromatographer (Thermo Fisher Scientific, Austin, TX) coupled to a mass spectrometer (Polaris Q, Thermo Fisher Scientific) (GC-MS). Samples were separated in a SP-2380 fused silica column (30 m x 250 µm x 0.20 µm, Supelco, Sigma-Aldrich). The injector was set at 200ºC, and the following gradient was used: 70°C for 5 min, followed by 4°C/min up to 140°C, 2°C/min up to 185°C, and 185°C for 10 min. Helium was used as the carrier gas, with a flow rate of 1 ml/min. The molecules were ionized by electron impact at 70 eV and 200 ºC. The spectra were collected in the 30–400 *m/z* range, and a chromatogram was generated by plotting the spectra of diagnostic fragment-ion species for *m/z* 41, 43, and 55. Fatty-acid species were identified by comparison with the FAME 37 methylated FA mix standard (Supelco, Sigma-Aldrich).

2.2. Results

2.2.1. Mass spectrometric analysis reveals phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine are newly generated phospholipids in Giardia

Several reports suggest that dietary lipids (i.e., PLs, fatty acids, and bile acids) are important for giardial growth and encystation in the human small intestine (Das et al. 2002). Reports also indicate that many of these lipids are not synthesized *de novo*; instead, they are scavenged from outside sources. To further elucidate whether giardial lipid compositions are similar to the growth medium or *Giardia* has the capacity to synthesize new lipids, the total lipid composition of vegetative and encysting trophozoites, and water-resistant cysts was determined by ESI-QTOF-MS (Figure 6A, B). The analysis in positive-ion mode identified multiple species of sphingomyelin (SM) and PC (Fig. 6A, B). Giardial PCs were composed mostly of palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids (Table 1). A few lipid species had the highest peak heights and were present in both vegetative and encysting trophozoites, as well as in *in vitro*-derived cysts — i.e., C16:0/d18:1-SM, C16:0/C18:1-PC, C18:1/C18:2-PC, C18:1/C18:1-PC, and C18:0/C18:1-PC. 18:1/18:1-PC appeared to be the major species (Figures 6A and B, 7A and B, Table 1). Supplemental Fig. 1A shows the fragmentation of the major PC species (C18:1/C18:1-PC) observed at *m/z* 792.6 (Fig. 6A). Loss of the choline head group is identified by the fragment ion at *m/z* 603.7, and loss of the acyl chains by ions at *m/z* 451.5 and 504.6 (Figure 7A).

Negative-ion mode spectra revealed that PGs are predominant PLs in *Giardia* not only in vegetative trophozoites but also in encysting cells and cysts. The phospholipids found include

primarily PG but also PE, PC, and two species of phosphatidylinositol (PI) (Figures 6B, 7C, 7D, 7E, Table 1). Interestingly, no phosphatidylserine (PS) could be detected. The fatty acids covalently linked to these PLs were similar to those found in positive-ion mode, with C16:0, C16:1, C18:0, and C18:1 fatty acids being the most predominant ones. In both negative- and positive-ion modes, we found that some of these fatty acids included species with retention time similar or identical to odd-carbon number fatty acids (OCFAs) (i.e., C15:0, C17:0, C17:1, C19:0) (Table 1). Since the GC-MS analysis was carried out using internal standards containing only linear OCFAs and a column that was not appropriate for discriminating between linear and monomethyl-branched OCFAs, we could not determine here the true nature of giardial OCFAs; thus, further experiments are required.

Moreover, it is possible sometimes to misidentify PG as lysobisphosphatidic acid (LBPA) or even other glycerophospholipids, because they share the same dehydrated glycerophosphate fragment-ion at *m/z* 153. In the current study, however, some characteristic fragment ions led to the unambiguous identification of these ion species as PGs (Boettner et al. 2005). For instance, Figure 7C shows fragmentation of the parent-ion species at *m/z* 749.7, tentatively assigned as C18:0/C16:0-diacyl-PG. The phospholipid was unequivocally identified as PG by the presence of the daughter-ion at *m/z* 675.5 ($[M - glycerol - H]^-$), which is derived from the neutral loss of the glycerol moiety linked to phosphate at the headgroup. The resulting fragment represents a phosphatidic acid with two fatty acids attached and, therefore, it is specific of PG and could not be generated from a lysobisphosphatidic acid (LBPA) species, because in the latter only one fatty acid is linked to each glycerol moiety. We also observed a fragment-ion at *m/z* 689.8, which most likely resulted from the internal fragmentation of the double-bond formed between C-2 and C-3 by dehydration of the glycerol backbone at the headgroup. Further fragmentation analysis of

m/z 749.7 confirmed the presence of C18:0 (*m/z* 283.3) and C16:0 (*m/z* 255.3) at *sn-1* and *sn-2*, respectively. The position of the *sn-1* and *sn-2* fatty acids was determined by their relative peak heights obtained by low collision energy dissociation, as reviewed by Pulfer and Murphy (Pulfer and Murphy 2003). Other fragments corresponding to the neutral loss of these fatty acids were also observed at *m/z* 465.4 ($[M - C18:0 - H]^-$), 483.4 ($[M - C18:0 + H_2O - H]^-$), 493.5 ($[M - C16:0 - H]^-$), and 511.5 ($[M - C16:0 + H_2O - H]^-$). The glycerol head group was confirmed by the formation of daughter-ions at *m/z* 391.3 and 419.3, which represent the loss of the *sn-1* and *sn-2* fatty acids, respectively, plus the glycerol moiety. A similar pattern of fragmentation was observed for other PG species identified in this study (Tables 1 and 2).

Earlier Ellis et al. (1996) have reported that PC, SM, and PE were the major phospholipids in *Giardia*, and that PG comprises only 10% of the total PL content in trophozoites (Ellis et al. 1996). In this study, however, the peak intensities and the number of PG species present in negative-ion mode indicate that PG could be a major phospholipid in this protozoan parasite (Figure 6C). As far as PE is concerned, we have found that this particular phospholipid is bound to common fatty acids such as oleate, linoleate, and stearate. Furthermore, the peak heights for PE species are higher than 50% in the full-scan spectra, indicating that these are a fairly abundant species (Table 1, and Figure 6B).

When a relative quantitative analysis of phospholipids was performed in positive-ion mode (Figure 8A), the major PC species observed was 18:1/18:1-PC (*m/z* 792.6), with higher (~75%) relative abundance observed in non-encysting trophozoites, and lower (~25%) in 12-h encysting cells. In cysts, nevertheless, the relative abundance of 18:1/18:1-PC returned to levels comparable to that of trophozoites. Another point of interest is the observation of C16:0/d18:1-SM at *m/z* 709.6 (Figure 8A). While the relative abundance of this sphingosine-based

phospholipid in trophozoites was <10% or even undetectable in 12-h encysting cells, it increased to ~25% in cysts, suggesting that SM might be important for cyst wall synthesis (Hernandez et al. 2008). For negative-ion mode analysis, the most predominant PL species observed was C18:0/C16:0-PG (*m/z* 749.5), with a relative abundance of 85%, as compared to 30% for the C12:0/C12:0-PE internal standard (*m/z* 578.4). Nevertheless, 18:0/16:0-PG decreased to ~45% in 12-h encysting cells and cysts. Other abundant PLs of interest in negative-ion mode, particularly in trophozoites, were 16:0/16:0-PG and 18:0/14:0-PG (both at *m/z* 721.5), with relative abundance of ~25%, making it clear that while the most commonly bound fatty acids were C16:0, C18:0, and C18:1, shorter fatty acids such as C14:0 were also present in reasonable amounts.

Giardia is cultured in TYI-S-33 media supplemented with adult bovine serum as a source of lipids. Bovine bile is also added to the media and is thought to facilitate the growth of *G. lamblia* by a mechanism yet to be elucidated. Studies also suggest that bile acids are taken up by the parasite through carrier-dependent pathway and most likely involve transporting lipid molecules by forming mixed micelles (Das et al. 1997). In the current study, an attempt was made to analyze the PL composition of serum and bile to verify which lipids could be present in the culture medium, because previous reports suggest that the lipid composition of *Giardia* closely resembles that of its growth medium, especially the medium containing high bile content (Jarroll et al. 1981; Kaneda and Goutsu 1988; Mohareb et al. 1991; Ellis et al. 1996). Our mass spectrometric data, on the other hand, show that serum and bile are rich in PC, *lyso*-PC, and diacylglycerol (DAG) (Table 2), and do not contain other PLs. This difference could be due to the fact that thin-layer chromatography (TLC) was used to separate PLs (Ellis et al. 1996), rather than a more sensitive and powerful resolving tool like mass spectrometry. Mass spectrometry has

the advantage over TLC and other methodologies of being able to resolve complex lipids with much higher sensitivity. Because the growth medium lacks PG and PE, the current results support the idea that at least PG and PE are newly synthesized PLs in *Giardia* and most likely generated via remodeling reactions as proposed earlier (Das et al. 2001; Das et al. 2002)

2.2.2. GC-MS analysis of fatty acids

Like PLs, fatty acids are also important for giardial growth and encystation (Das et al. 2001). Although fatty acids trigger encystation in vitro (Gillin et al. 1987), excess fatty acids are toxic and possess anti-giardial properties. It has been demonstrated that medium-chain fatty acids, especially dodecanoic acid (C12:0), kill *Giardia* in vitro with (LD_{50} ~13.78 µg/ml) (Rayan et al. 2005). Previous reports (Kaneda and Goutsu 1988; Mohareb et al. 1991; Ellis et al. 1996) indicate that C16:0 is the major fatty acid in vegetative and encysting *Giardia*, followed by C18:0, C18:1, and C18:2. Traces of C14:0, C15:0, C17:0, C18:3, C19:0, C20:0, C22:0, C24:0, C26:0, and C28:0 have also been detected. Interestingly, no dramatic differences were observed in fatty-acid compositions between vegetative and encysting trophozoites. In the current study, the total fatty acid content of trophozoites, encysting cells, and cysts was analyzed by GC-MS, and the results revealed that C16:0, C18:0, and C18:1 were indeed the major fatty acids in all giardial lipid species, as evidenced by relative peak areas (Figure 9A). However, less common acyl groups such as C10:0, C12:0 and C14:0 were also present. The fatty-acid composition remained unchanged in vegetative trophozoites, encysting cells (0–48 hours), and water-resistant cysts (Figure 9A, Table 3). In serum and bile, the major fatty acids are C16:0, C18:0, C18:1, and C18: 2 (Figure 9B, Table 3). Traces of other fatty acids such as C12:0, C14:0, C15:0, C17:0,

C20:0, and C22:0, were also identified. Our results also suggest that some long chain fatty acids (i.e., C24:0, C24:1, C23:0, etc.), which are present in *Giardia,* could be synthesized *de novo* by the action of elongase(s). Although no attempt was made to measure the elongase activity, the BLAST search of the *Giardia* Genome Database (http://giardiadb.org/giardiadb/) (Morrison et al. 2007) yielded the presence of fatty acid elongase 1 gene (accession no. XM_00170849.1, E-value 4e-81), suggesting that fatty acid elongation machinery may be present in this pathogen. Interestingly, in *Dictyostelium discoideum*, the same sequence was annotated as fatty acid elongase 3-ketoacyl-CoA synthase (NCBI accession no. XP_638938). Our GC-MS analysis also revealed that the sterol fractions purified from trophozoites, encysting cells and cysts contain only cholesterol (Figure 10). However, a previous report suggests the presence of low levels of ergosterol along with the more abundant cholesterol in this protozoan parasite (Ellis et al. 1996).

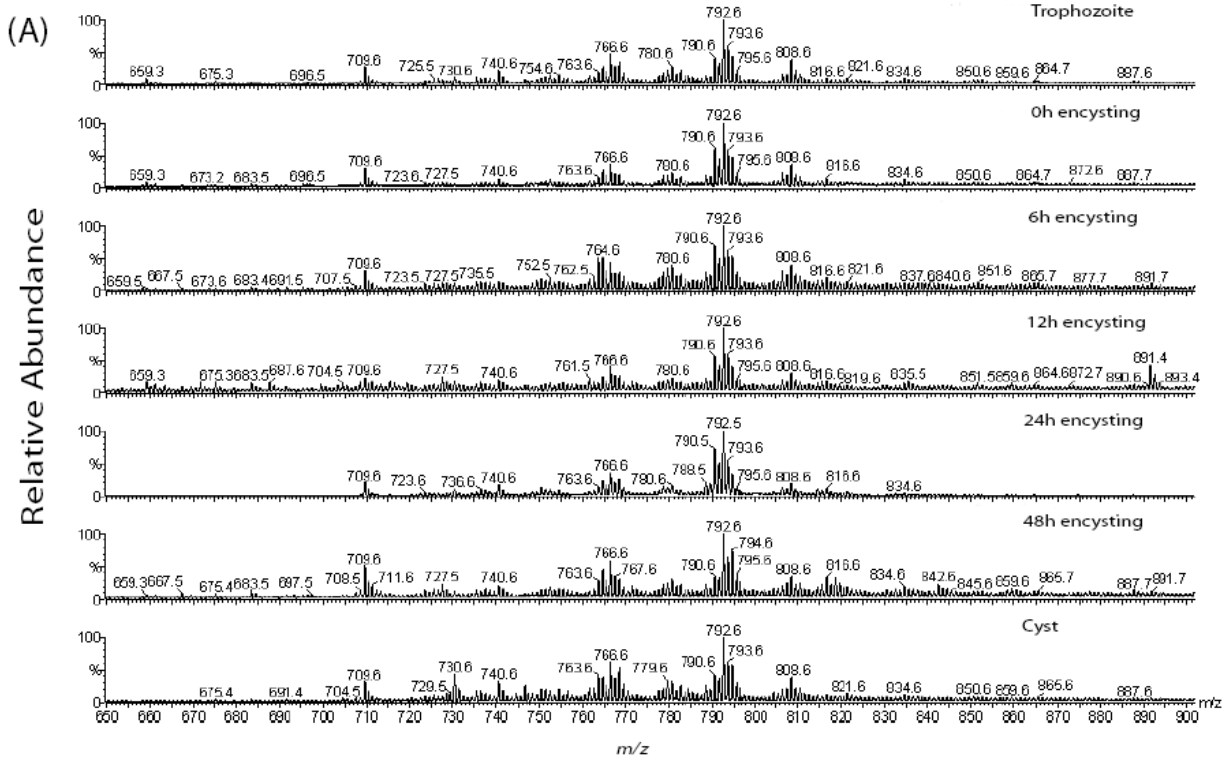
2.3. Conclusions

The current lipidomic analysis generated valuable information regarding the metabolism and synthesis of phospholipids in *Giardia*. It was observed that giardial lipid composition was different than the lipid and fatty-acid contents of the growth medium, and that PG and PE are newly generated phospholipids, which suggests that this evolutionarily basal organism may use a sophisticated pathway to carry out lipid synthesis. In addition to PG and PE, I also detected new fatty acids, which are not present in the medium. Interestingly, the *Giardia* genome database (www.GiardiaDB.org) did not reveal the existence of well-known fatty-acid biosynthesis pathways that are operative in bacteria and mammalian cells, which further indicated its

evolutionary position—i.e., deep in the branch leading from prokaryotes to higher eukaryotes (Sogin et al. 1989). The presence of mono-methylated branched-chain fatty acids (MBCFA) further supports the primitive metabolic characteristics of this unicellular protozoan because MBCFAs are ubiquitous in bacteria and archaea but are not present in mammalian cells (Chattopadhyay and Jagannadham 2003). Thus, the lipid metabolic abilities of this organism are quite varied, with some being very primitive and others being more evolved than previously reported.

The analysis presented in the current dissertation also emphasizes the use of sophisticated instruments, and so analyzing software should be employed to generate more accurate descriptions of any biological processes. The mass spectrometric analysis of the giardial lipidome reveals the presence of many PL and fatty-acid species that could not be detected earlier by TLC and HPLC. Detection of new lipid and fatty-acid species in *Giardia* encouraged me to investigate the pathways that allow this parasite to synthesize new phospholipids by genomic and biochemical analyses described in Chapter 3 and Chapter 4.

Figure 6


(A)
Relative Abundance
Trophozoite
0h encysting
6h encysting
12h encysting
24h encysting
48h encysting
Cyst
m/z

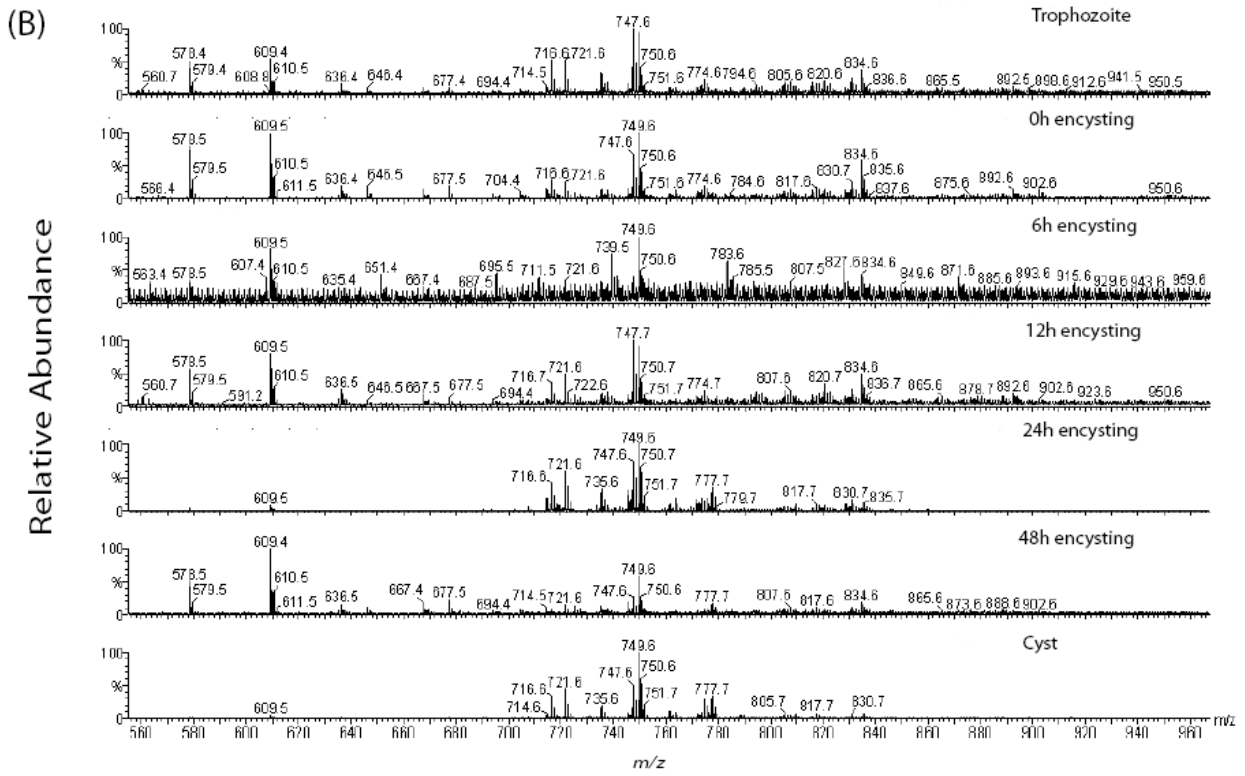
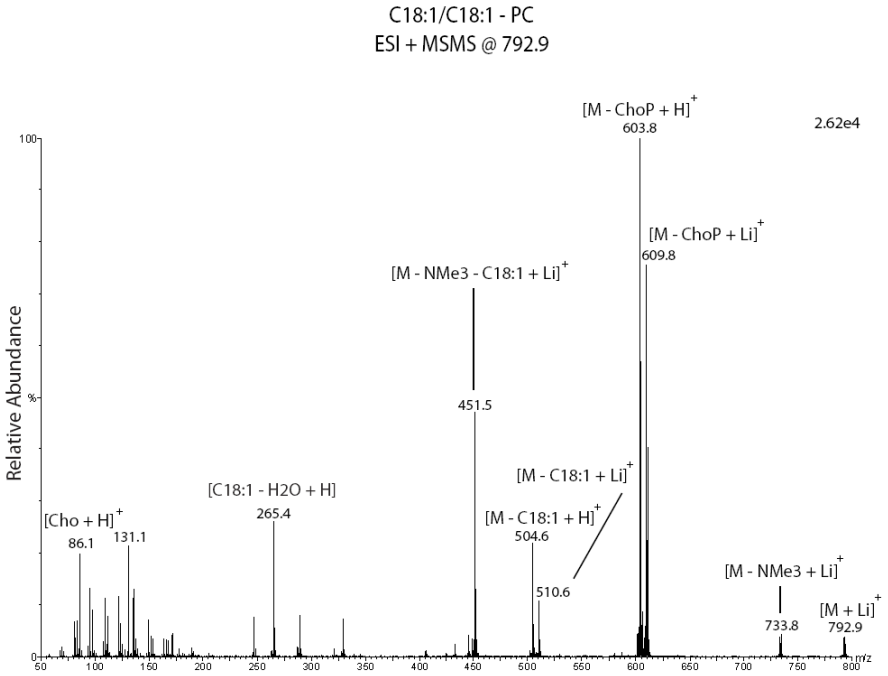


Figure 6. Lipid analysis by MS of *G. lamblia*. Total phospholipids were fractionated by silica-gel 60 and analyzed by ESI-QTOF-MS. (A and B) Positive- and negative-ion mode full-scan spectra of giardial phospholipids, respectively. Total lipids and phospholipids from vegetative, encysting and water-resistant cysts were isolated as described in the Materials and Methods. (Yichoy et al. 2009).

Figure 7

(A)


C18:1/C18:1 - PC
ESI + MSMS @ 792.9
2.62e4
Relative Abundance
[M - ChoP + H]+
603.8
[M - ChoP + Li]+
609.8
[M - NMe3 - C18:1 + Li]+
451.5
[M - C18:1 + Li]+
[C18:1 - H2O + H]
265.4
[Cho + H]+
86.1
131.1
[M - C18:1 + H]+
504.6
510.6
[M - NMe3 + Li]+
733.8
[M + Li]+
792.9
m/z

(B)

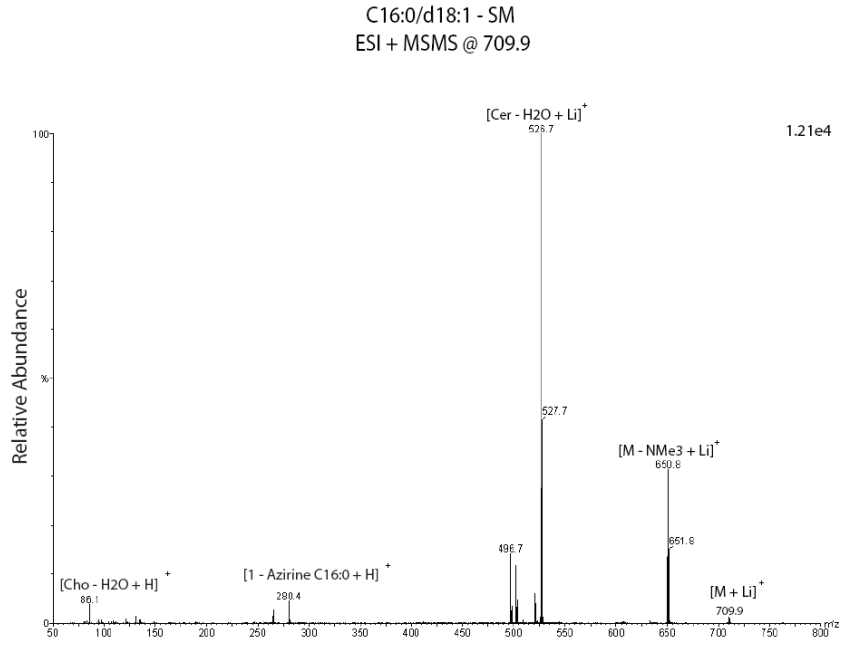
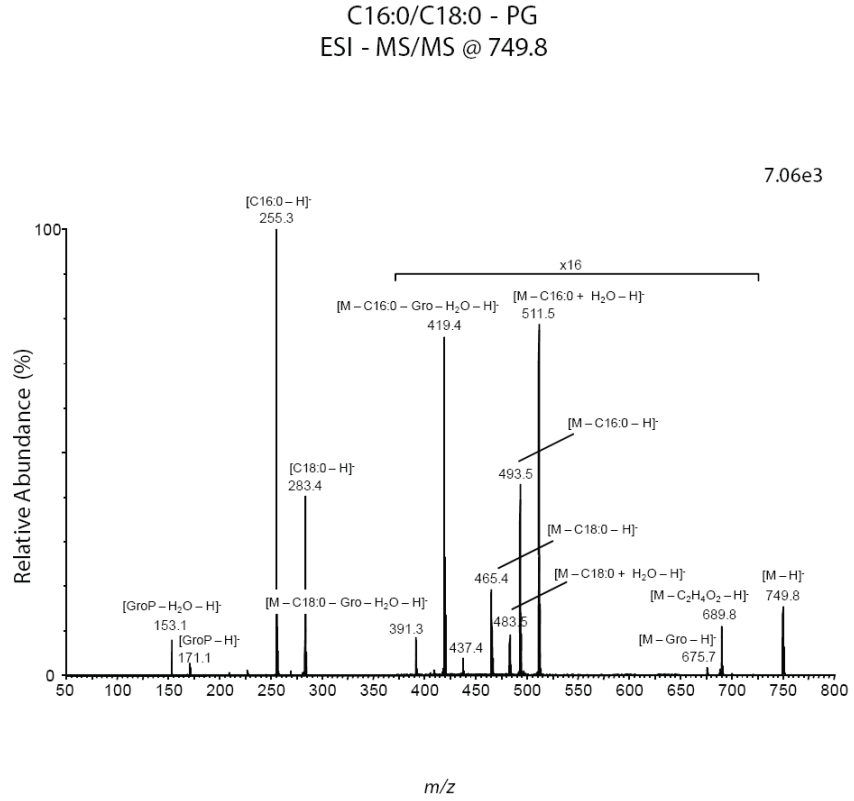
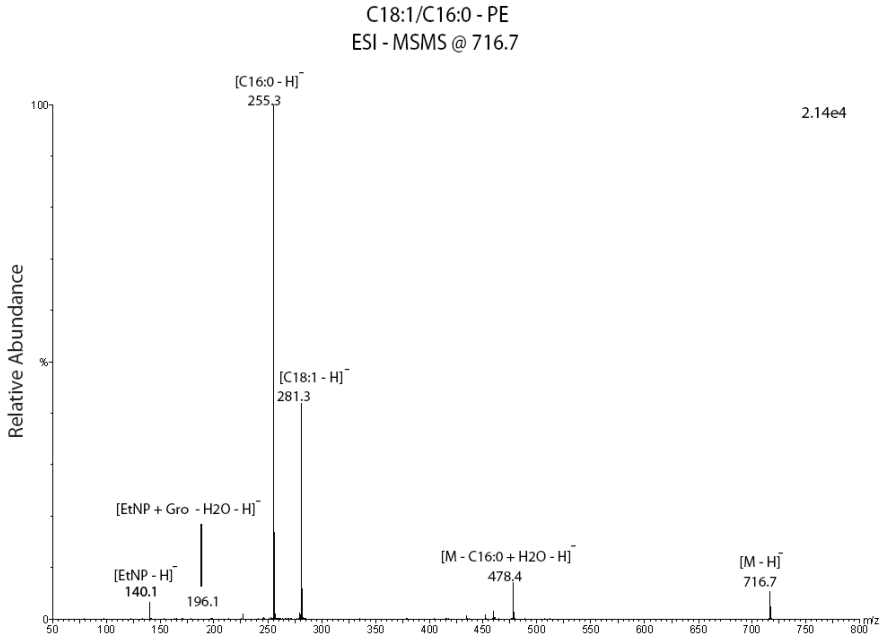

C16:0/d18:1 - SM
ESI + MSMS @ 709.9
1.21e4
Relative Abundance
[Cer - H2O + Li]+
526.7
527.7
[M - NMe3 + Li]+
650.8
651.8
496.7
[Cho - H2O + H]+
86.1
[1 - Azirine C16:0 + H]+
280.4
[M + Li]+
709.9
m/z

Figure 7

(C)


C16:0/C18:0 - PG
ESI - MS/MS @ 749.8
7.06e3
Relative Abundance (%)
m/z
[C16:0 – H]-
255.3
x16
[M – C16:0 – Gro – H2O – H]-
419.4
[M – C16:0 + H2O – H]-
511.5
[M – C16:0 – H]-
493.5
[C18:0 – H]-
283.4
[M – C18:0 – H]-
465.4
[M – C18:0 + H2O – H]-
483.5
[M – C2H4O2 – H]-
689.8
[M – H]-
749.8
[GroP – H2O – H]-
153.1
[GroP – H]-
171.1
[M – C18:0 – Gro – H2O – H]-
391.3
437.4
[M – Gro – H]-
675.7

(D)


C18:1/C16:0 - PE
ESI - MSMS @ 716.7
2.14e4
Relative Abundance
m/z
[C16:0 - H]-
255.3
[C18:1 - H]-
281.3
[EtNP + Gro - H2O - H]-
196.1
[EtNP - H]-
140.1
[M - C16:0 + H2O - H]-
478.4
[M - H]-
716.7

(E)

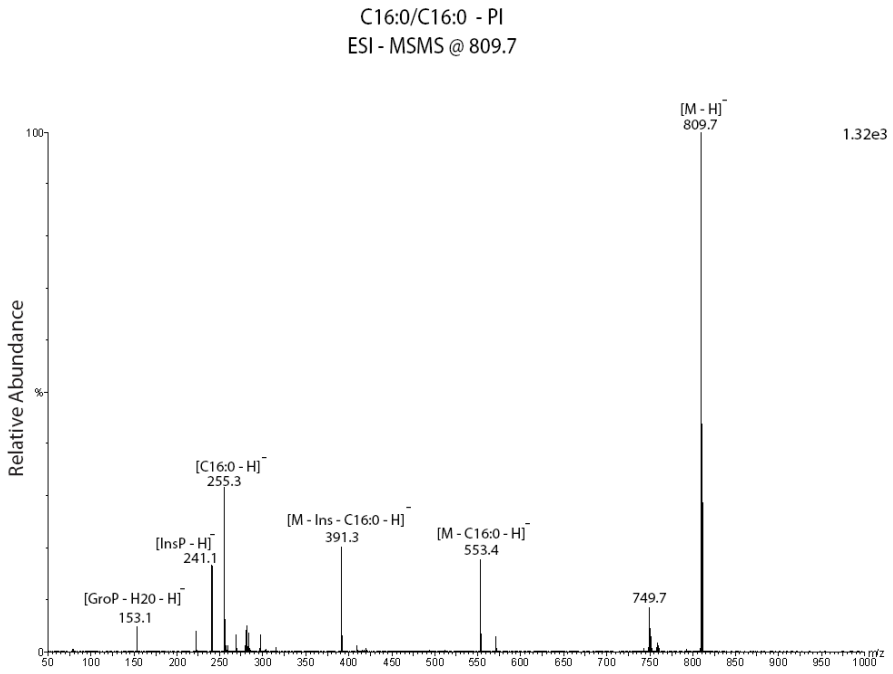


Figure 7. Lipid analysis by MS of *G. lamblia*. Total phospholipids were fractionated by silica-gel 60 and analyzed by ESI-QTOF-MS. (A and B) Positive-ion mode MS-MS spectra of C18:1/18:1-PC (*m/z* 792.7) C16:0/d16:1-SM at *m/z* 709.9, respectively. (C) MS-MS spectrum of C18:0/16:0-PG parent-ion at *m/z* 749.5, ionized in negative-ion mode. "x16" indicates that the portion of that spectrum was magnified sixteen times to make the peaks more visible. The number at the top right corner of each spectrum indicates signal strength, measured as ion intensity at 100% relative abundance. *m/z*, mass to charge ratio. (D) Negative-ion mode MS-MS spectra of C18:1/C16:0-PE (*m/z* 716.7). (E) C16:0/C16:0-PI (*m/z* 809.7), respectively. The number at the top right corner of each spectrum indicates signal strength, measured as ion intensity at 100% relative abundance. *m/z*, mass to charge ratio (Yichoy et al. 2009).

Table 1. Lipid analysis and composition of the major phospholipids from differentiating *Giardia lamblia* (Yichoy et al. 2009). [a]

[a] Phospholipid species were identified by MS-MS analysis in positive- and negative-ion modes.

[b] Relative abundance is designated by the peak height: ++++, up to 100%; +++, up to 75%; ++, up to 50%; +, 10% or less. IS, internal standard.

[c] Relative abundances for each sample were similar and therefore only the peak heights for trophozoites are shown. N/A, not applicable.

PL	*m/z*	Ion Species	Proposed Structure *sn-1/sn-2* [b]	Relative Abundance [c]
Positive-Ion Mode				
PC	502.4	M + Li	*lyso*-C16:0	+
	526.4	M + Li	*lyso*-C18:2	+
	528.4	M + Li	*lyso*-C18:1	+
	600.5	M + Li	C11:0/C11:0 (IS)	N/A
	740.8	M + Li	C16:0/C16:0	+
	752.7	M + Li	C16:0/C17:1 and/or C18:1/C15:0	+
	754.8	M + Li	C16:0/C17:1	+
	764.8	M + Li	C16:1/C18:1	+
	766.8	M + Li	C16:0/C18:1	++
	768.8	M + Li	C16:0/C18:0	+
	780.6	M + Li	C17:0 /C18:1	+
	788.6	M + Li	C18:2/C18:2	+
	790.7	M + Li	C18:1/C18:2	+
	792.7	M + Li	C18:1/C18:1	++
	794.6	M + Li	C18:0/C18:1	++++
	806.8	M + Na	C18:1/C18:2	+
	808.8	M + Na	C18:1/C18:1	++
	814.6	M + Li	C20:4/C18:1	++
	816.6	M + Li	C20:4/C18:0	+
SM	709.9	M + Li	C16:0/d18:1	++
	725.7	M + Na	C16:0/d18:1	+
	737.8	M + Li	C18:0/d18:1	+

Table 1. Continued.

PL	*m/z*	Ion Species	Proposed Structures *sn-1/sn-2* [b]	Relative Abundance [c]
Negative-Ion Mode				
PC	804.6	M + formate	C18:1/C16:0	+
	806.6	M + formate	C18:0/C16:0	+
	820.6	M + Cl	C18:1/C18:1	+
	828.6	M + formate	C18:2/C18:1	+
	830.6	M + formate	C18:1/C18:1	+
	832.6	M - H	C18:0/C18:1	+
PE	578.4	M - H	C12:0/C12:0 (IS)	N/A
	636.4	M + NaCl - H	C12:0/C12:0 (IS)	N/A
	646.4	M + Na + formate - H	C12:0/C12:0 (IS)	N/A
	714.5	M - H	C16:0/C18:2 and/or C16:1/C18:1	++
	716.5	M - H	C18:1/C16:0	+++
	834.6	M - H	C22:6/C22:6 (IS)	N/A
PG	609.4	M - H	C12:0/C12:0 (IS)	N/A
	707.5	M - H	C16:0/15:0 and/or C14:0/C17:0	+
	719.6	M - H	C16:0/C16:1 and/or C18:1/C14:0	+
	721.5	M - H	C16:0/16:0 and/or C18:0/C14:0	+++
	733.5	M - H	C16:0/C17:1 and/or C18:1/C15:0	+
	735.5	M - H	C16:0/C17:0 and/or C15:0/C18:0	+
	745.5	M - H	C18:2/C16:0 and/or C16:1/C18:1	+
	747.5	M - H	C18:1/C16:0	++++
	749.5	M - H	C18:0/C16:0	++++
	761.5	M - H	C18:1/C17:0	+
	763.6	M - H	C18:0/C17:0 and/or C19:0/C16:0	+
	771.5	M - H	C18:1/C18:2	+
	773.5	M - H	C18:1/C18:1	+
	775.5	M - H	C18:0/C18:1	+
	777.6	M - H	C16:0/20:0 and/or C18:0/C18:0	+
	789.5	M + Na + formate – H	C16:0/C16:0, C18:0/C14:0, and/or C15:0/C17:0	+
	817.5	M + Na + formate – H	C18:0/C16:0	+
PI	809.5	M - H	C16:0/C16:0	+
	835.5	M - H	C18:1/C16:0	+

Table 2. Positive-ion mode MS-MS analysis of phospholipids from bile and serum (Yichoy et al. 2009).

m/z	**Ion Species**	**Proposed Structures** ***sn-1/sn-2***
526.4	M + Li	*lyso*-C18:2-PC
528.4	M + Li	*lyso*-C18:1-PC
530.5	M + Li	*lyso*-C18:0-PC
552.4	M + Li	*lyso*-C20:3-PC
653.7	M + Li	C18:0/C20:3-DAG
764.7	M + Li	C16:0/C18:2-PC
766.7	M + Li	C16:0/C18:1-PC
790.7	M + Li	C18:1/C18:2-PC and/or C18:0/C18:3-PC
792.7	M + Li	18:0/18:2-PC
794.7	M + Li	18:0/18:0-PC
816.7	M + Li	18:0/20:4-PC

Figure 8

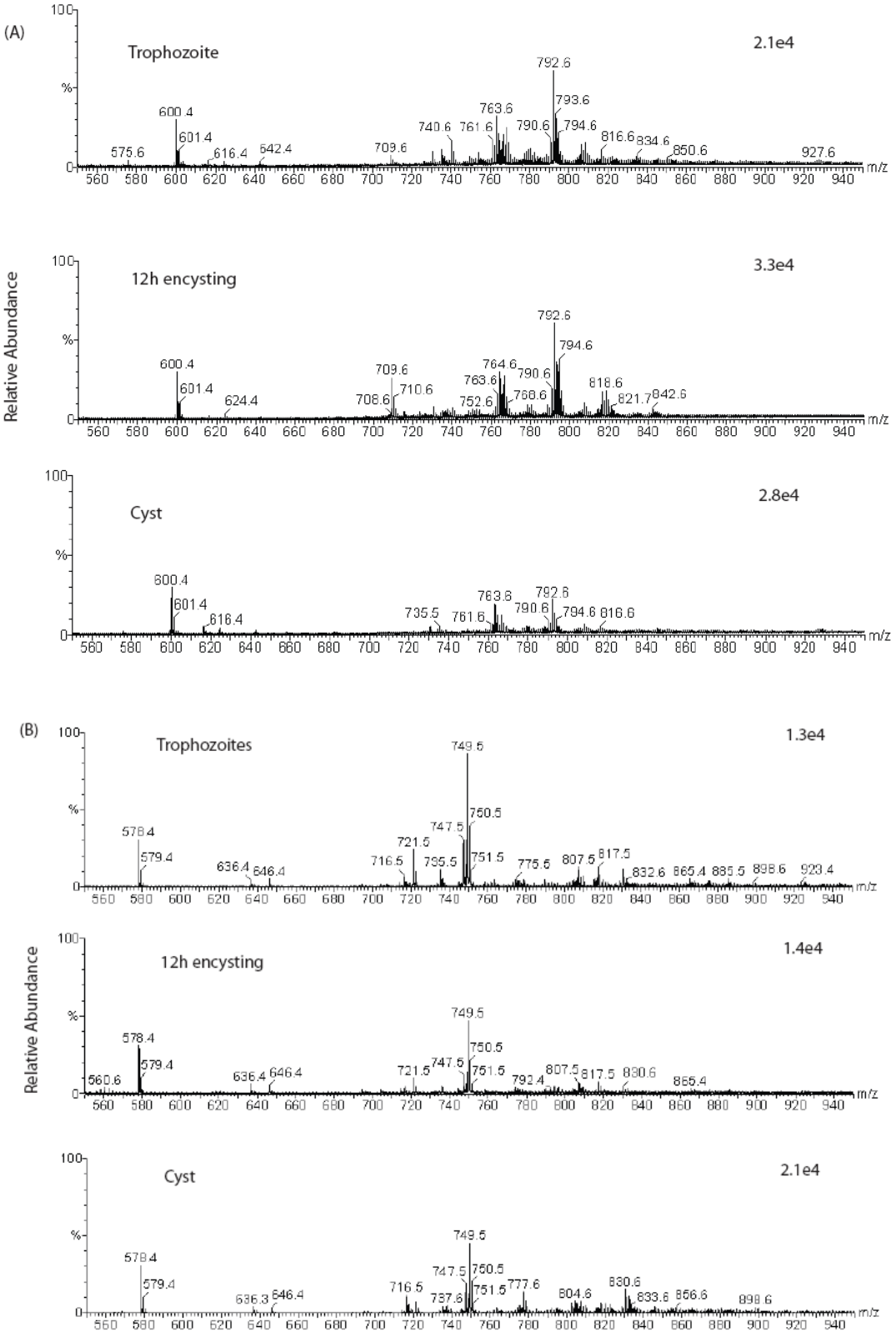

(A)
Trophozoite
2.1e4
12h encysting
3.3e4
Cyst
2.8e4
Relative Abundance
m/z
(B)
Trophozoites
1.3e4
12h encysting
1.4e4
Cyst
2.1e4

Figure 8. ESI-QTOF-MS spectra of relative quantitative analysis of giardial phospholipids. Total lipids were extracted and fractionated as described in Materials and Methods. Cell numbers were adjusted to 5000 cells/μL. (A and B) Positive- and negative-ion mode analysis, respectively. For positive-ion (ESI+) mode MS analysis, giardial lipid samples were spiked with 2.5 μM 11:0/11:0-PC (*m/z* 600.4) ($[M + Li]^+$), used as internal standard (IS). For negative-ion (ESI-) mode MS analysis, 5 μM 12:0/12:0-PE (*m/z* 578.4) ($[M - H]^-$) was used as the IS. *m/z*, mass to charge ratio (Yichoy et al. 2009).

Figure 9

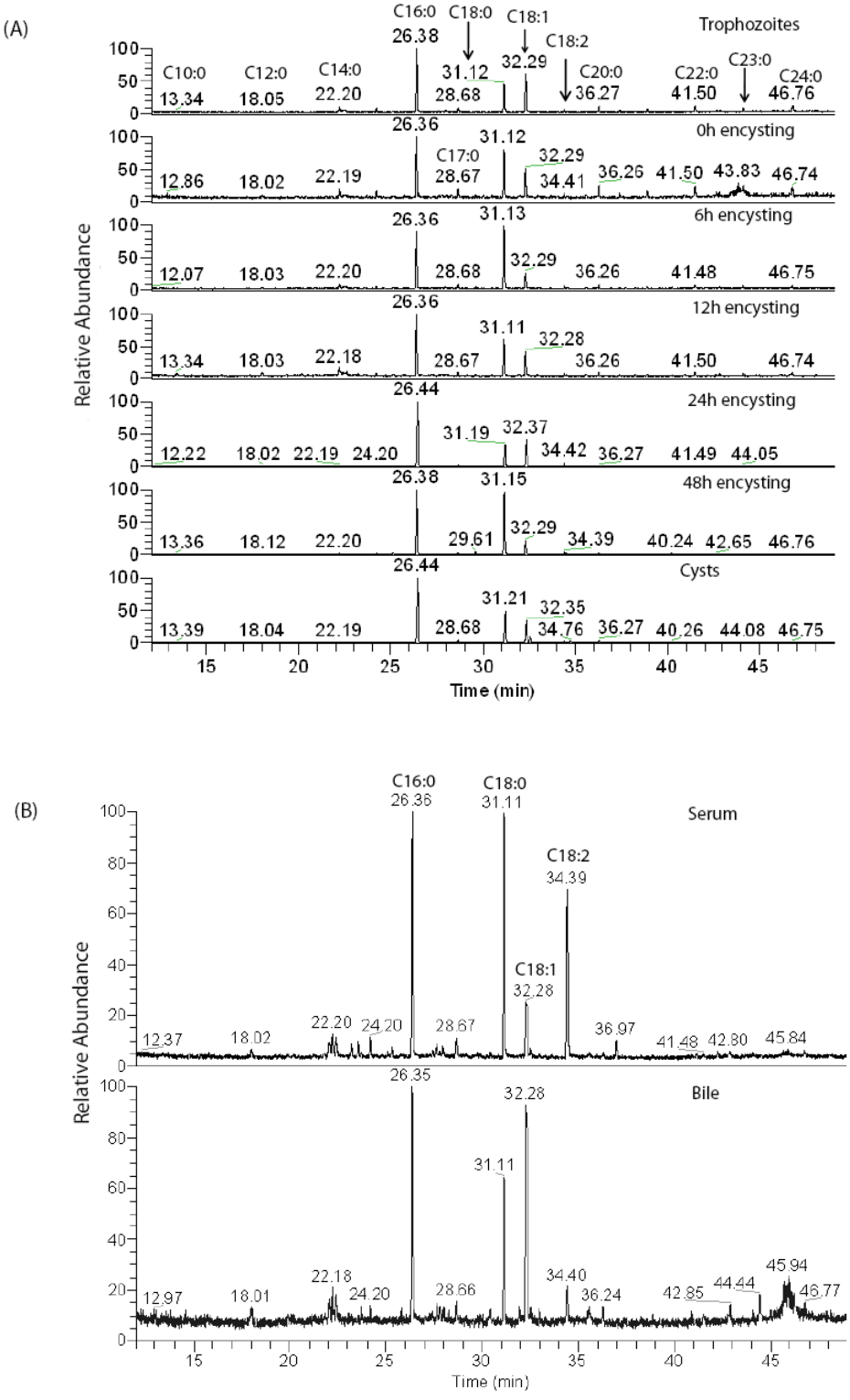

(A)
Trophozoites
C16:0
C18:0
C18:1
C18:2
C20:0
C22:0
C23:0
C24:0
C10:0
C12:0
C14:0
C17:0
0h encysting
6h encysting
12h encysting
24h encysting
48h encysting
Cysts
Relative Abundance
Time (min)
(B)
Serum
Bile
Relative Abundance
Time (min)

Figure 9. GC-MS spectra of fatty acid content. Total lipids from vegetative trophozoites, encysting cells, water-resistant cysts, bile and serum were isolated and processed as described in Materials and Methods. (A) Giardial fatty acids; (B) Fatty acids from the bile and serum. The retention times (min) of identified fatty acids and internal standards are indicated (Yichoy et al. 2009).

Table 3. Fatty acid analysis by GC-MS (Yichoy et al. 2009). [a]

[a] Fatty acids present in trophozoites, encysting cells, in vitro-derived cysts, and bovine bile and serum.

[b] Relative abundance is designated by peak area and is shown as percentage of the total fatty acid content for each sample.

Retention time (min)	Fatty acid	Relative Peak Area (%) [b]								
		Vegetative Trophozoites	0-h Encysting	6-h Encysting	12-h Encysting	24-h Encysting	48-h Encysting	In vitro Cysts	Bovine Serum	Bovine Bile
13.3	C10:0	0.0	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0
18.0	C12:0	1.1	1.9	0.0	2.7	0.0	0.7	0.4	1.7	3.2
22.2	C14:0	3.6	3.4	2.7	6.1	0.3	0.8	0.3	2.8	3.2
24.2	C15:0	1.6	2.0	1.3	1.3	0.4	0.6	0.3	2.3	trace
26.3	C16:0	33.5	25.7	32.2	33.3	51.7	38.2	47.7	27.4	30.2
28.6	C17:0	2.6	3.6	2.7	2.0	1.1	0.9	1.5	2.7	2.1
31.1	C18:0	15.9	22.3	39.1	23.4	19.1	45.0	27.6	30.9	22.2
32.2	C18:1	22.7	12.2	10.8	16.1	23.7	9.2	18.2	7.7	31.4
34.4	C18:2	1.3	1.5	2.0	1.8	2.7	2.3	1.3	24.5	5.7
36.2	C20:0	3.5	6.6	2.7	2.7	0.9	1.3	2.1	trace	2.0
37.4	C20:1	0.9	1.4	0.0	0.0	0.0	0.1	0.1	0.0	0.0
38.8	C21:0	1.9	2.7	1.1	1.1	0.0	0.0	0.0	0.0	0.0
41.5	C22:0	3.9	5.6	2.6	2.8	0.1	0.5	0.3	0.0	0.0
44.0	C23:0	2.3	6.2	1.1	1.7	0.0	0.2	0.1	0.0	0.0
46.7	C24:0	4.4	4.9	1.7	2.9	0.0	0.2	0.1	0.0	0.0
48.1	C24:1	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

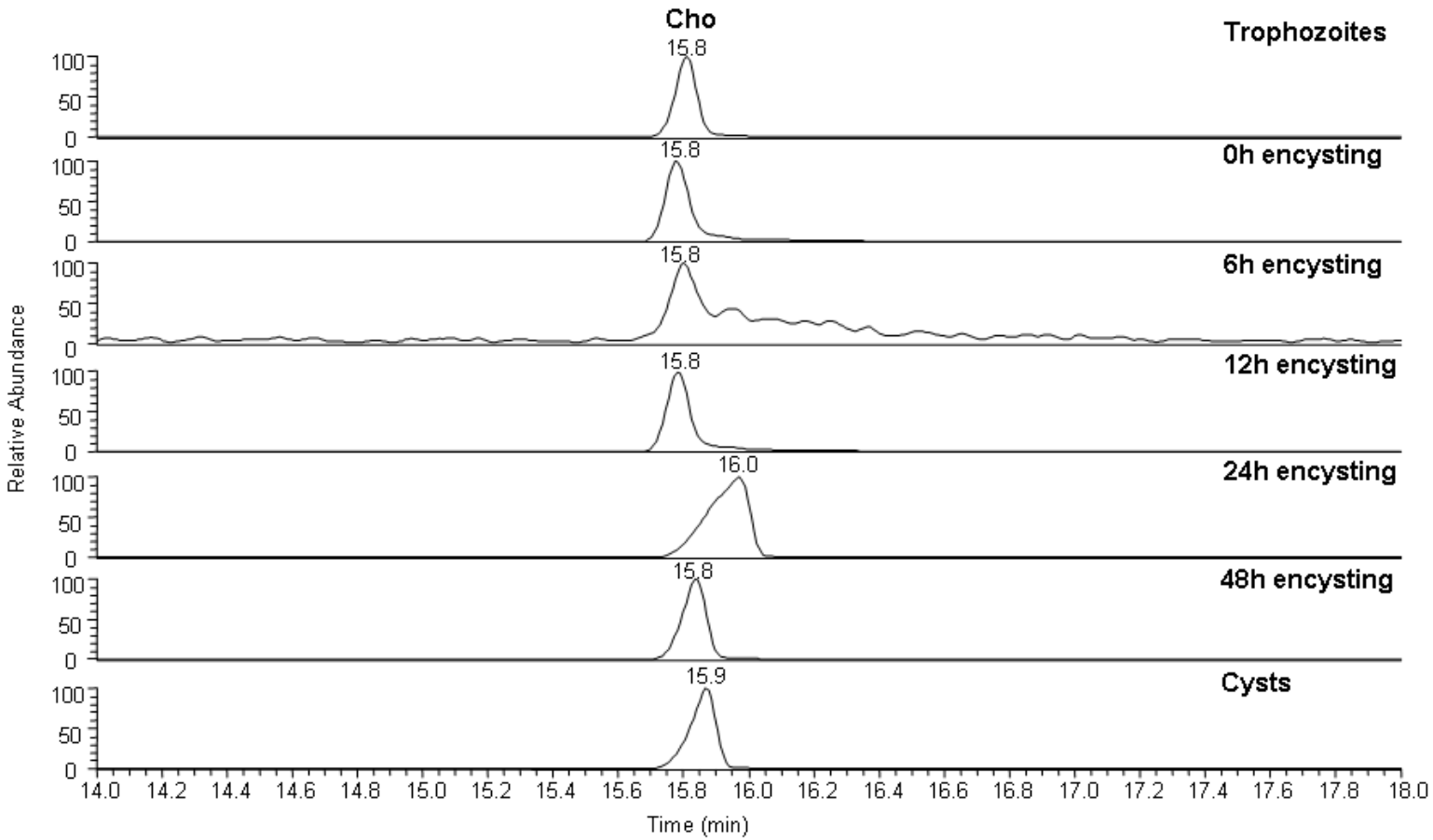


Figure 10. Sterol analysis by GC-MS. Neutral lipids eluted with choloroform from total lipid extract
Cho: cholesterol (Yichoy et al. 2009)

Chapter 3: Genomic and transcriptional analyses of putative phospholipid metabolic and transporter genes

The goal of this chapter is to identify the genes of lipid metabolic and transporter enzymes present in *Giardia.* The data presented in Chapter 2 indicates that PG and PE are most likely newly generated phospholipids (PLs) and that they are not obtained from the culture media (Yichoy et al. 2009). Determining whether the *Giardia* genome contains the open-reading frames (ORFs) for PL synthesis, metabolism, and transport proteins will provide important information regarding lipid biochemistry of this protozoan parasite. Because of the fact that the giardial growth medium contains only PC and *lyso*-PC (discussed in Chapter 2), and due to the fact that *Giardia* has a limited ability to synthesize its own lipids (Jarroll et al. 1981), it is conceivable that PC/lyso-PC serve as precursors of PG and PE. Most likely the lipids from the growth medium are internalized by lipid transporters (i.e., flippase) and remodeled by head-group or base-exchange enzymes such as phosphatidylglycerolphosphate synthase (PGPS) and phosphatidylserine decarboxylase (PSD). The focus of Chapter 3 is to carry out various bioinformatic (i.e., Pfam, phylogeny, predicted cellular localizations, topology, etc.) analyses, along with SYBR-Green assisted transcriptional assays of *pgps* and *psd* to comprehend the nature and functions of these lipid biosynthesis and transport genes in *Giardia*. Such analyses should predict considerable information with regard to the lipid metabolomics of this early divergent eukaryote.

3.1. Materials and Methods

Predicted open-reading frames (ORFs) were obtained from the *Giardia* genome database (http://giardiadb.org/giardiadb/) and were compared using BLASTP (Morrison et al. 2007). Sequences for PE and PG biosynthesis were identified, including the genes of phosphatidylserine decarboxylase (*psd,* accession no. XM_001707858, ORF no. 16495) and phosphatidylglycerolphosphate synthase (*pgps*, accession no. XP_769290, ORF no. 7259). PCR primers were designed using Primer3 software (http://frodo.wi.mit.edu) and were synthesized by MWG Biotech (High Point, NC) or Sigma Genosys (St. Louis, MO). The sequences of the primer pairs are as follows.

Following harvest, cysts were re-suspended in 4 ml TRI reagent (Sigma) and freeze-thawed at -20 °C three times. Following the last freeze-thaw, 0.5-mm glass beads (BioSpec Products) were added, and the cyst wall was broken by vortexing for approximately 5 min. Total RNA was extracted from trophozoites and encysting cells using TRI reagent and was reverse transcribed using the ImProm-II Reverse Transcriptase Kit (Promega). Levels of PGPS (GenBank accession number XP_769290) and PSD (GenBank accession number XM_001707858) transcription were quantified by quantitative, real-time PCR. RT^2 Real-Time SYBR Green/Fluorescein PCR Master Mix (SuperArray) was used in the reaction mixture, and all reactions were performed three times. Primers targeting α-tubulin were used as a control to normalize the samples. Transcript levels were quantified using the relative standard-curve method (Hernandez et al. 2007; Morrison et al. 2007). Statistical analysis was performed with one-way ANOVA with Dunnett's Post Test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

3.2. Results

3.2.1. Genomic analysis of PG and PE synthesis and Flippase genes

Because PG and PE are not present in bile or serum (Table 2), we asked whether they could be newly synthesized in *Giardia*. Therefore, we searched the *Giardia* Genome Database (http://giardiadb.org/giardiadb) (Morrison et al. 2007) to identify PG- and PE-related synthesis/remodeling genes. Furthermore, because it has been suggested that the *Giardia* genome has limited lipid-remodeling abilities and that many of the traditional *de novo* lipid synthesis enzymes are not found in the giardial genome, we asked whether this protozoan has flippases, or ATP-dependent phospholipid transporters (Daleke and Lyles 2000; Morrison et al. 2007). The BLASTp search yielded giardial phosphatidylglycerolphosphate synthase (*gpgps)* and phosphatidylserine decarboxylase (*gpsd*) genes, as well as four flippases. Pfam analyses revealed that *gpgps* matched well with bacterial and trichomonads *pgps* (see Table 4). The two closest matches were *Opitutaceae bacterium* and *Trichomonas vaginalis*, with E-values of 1.00 e-32 and 5.00 e-25, respectively. However, the open-reading frame (ORF) annotations (http://giardiadb.org/giardiadb/) (Morrison et al. 2007) for these matches were CDP-DAG-glycerol-3-phosphate and 3-phosphatidyltransferase, rather than PGPS, and they belong to the CDP-alcohol phosphatidyltransferase family of enzymes. On the other hand, *gpsd* yielded matches of the eukaryotic phylum, with the closest being *Plasmodium vivax* (E-value 2.00 e-22). Other protozoa with *psd* sequences that closely matched the giardial *psd* included *Toxoplasma gondii* and *Plasmodium falciparum*. For example, *gpsd* belongs to the phosphatidylserine decarboxylase family of enzymes. Unlike gPSD and gPGPS, three of the flippase protein sequences did not match to bacteria or other protozoans. Instead, these most closely match to

Homo sapiens (E-value 5 e-76 and 5 e-26) and *Rattus norvegicus* (E-value 4 e-94), while the last matches to *Pediculus humanis corporis* (2 e-94) as well as to *Entamoeba histolytica* (2 e-90). Pfam analysis shows that all four giardial flippases belong to the family of E1 and E2 ATPases (Table 4).

To retrieve the predicted sub-cellular localization of gPGPS and gPSD, DNA sequences were translated into protein using the translation tool, available at the Expert Protein Analysis System (ExPaSy) proteomics server (www.expasy.ch/) (Gasteiger et al. 2003; Morrison et al. 2007). The translated protein sequences were then entered into PSORTII to predict the sub-cellular localizations. PORTII predicts that gPGPS is an ER-bound protein and that gPSD localizes in the cytoplasm in *Giardia* (Horton and Nakai 1997; Nakai and Horton 1999) (Table 5). Because the PSORTII tool only has options for fungal, bacterial, plant and mammalian sequences, the CSS-Palm tool was employed to verify that membrane localization of the four flippases. Protein palmitoylation is a post-translational modification that acts as a membrane target anchor. Therefore, if the giardial flippases are indeed membrane-bound proteins, CSS-Palm would act as another prediction tool in addition to PSORT (Greaves and Chamberlain 2007; Emmer et al. 2009). The CSS-Palm tool indicates that all four flippases as well as PGPS and PSD have palmitoylation sites (Table 5).

3.2.2. Transcriptional analysis suggests that gpgps and gpsd genes are transcribed in Giardia

Recent results from our laboratory suggest that many lipid synthesis genes in *Giardia* are regulated differentially during encystation (Hernandez et al. 2008). Here, we asked whether *gpsd* and *gpgps* are transcribed and are expressed in trophozoites and cysts. Quantitative, reverse–

transcription, polymerase chain-reaction (qRT-PCR) results indicate that both *gpsd* and *gpgps* are expressed in vegetative and encysting trophozoites as well as in water-resistant cysts. It is clear from Figure 11 that these two genes display differential patterns of expression. For example, in 6-h and 12-h encysting cells, *gpsd* transcript levels decrease ~60% and 50%, respectively, whereas in 24-h encysting cells the transcript level is two-fold higher than in the vegetative state and remains high (~1.5 fold) in cysts. Transcript levels were found to be significantly different from vegetative trophozoites by one-way ANOVA with Dunnett's Post Test ($p < 0.05$). In the case of *gpgps*, the transcript levels decrease by ~30% in 6-, 12-, and 24-h encysting cells. In 48-h encysting cells and cysts, expression increases ~1.5 fold (Figure 10B).

Interestingly, Dunnett's Post Test did not show that *gpgps* transcript levels in encysting cells or cysts were significantly different from trophozoites ($p > 0.05$). Although it is not known whether these genes are translated into products, data indicate that both gPSD and gPGPS could play significant roles in giardial biology. For example, while gPSDs are required for encystation, they might play precise roles in forming cysts and during excystation to trophozoites.

3.3. Conclusions

Despite the fact that giardial PSD most closely resembles the protein sequence of *Plasmodium vivax*, it is unlikely that *Giardia* shares many similarities with the phospholipid metabolism of *Plasmodium*. While both sets of organisms are capable of generating PE by head-group exchange, the similarities end there (Vial et al. 2003). *Plasmodium* has more capabilities for generating phospholipids *de novo*, unlike *Giardia*. Also, the similarities between the giardial PGPS and bacterial homologues reiterates the position of this protozoan as an evolutionarily

basal organism and reinforces the fact that *Giardia* maintains metabolic similarities with both other eukaryotes and bacteria and *Archaea*. Furthermore, given that PG is primarily found in the plasma membrane of bacteria and mitochondrial membranes of eukaryotes, these similarities to bacteria are logical. It would be of interest to determine whether giardial PG localizes in the mitosome. Previously, it has been reported that PG localizes throughout the cytoplasm and in the perinuclear regions (Das et al. 2001), but it is not known whether these areas of fluorescence would co-localize with mitosome antibodies.

The Pfam matches for the four gFlip enzymes indicate that flippases are well conserved, despite the differences in lipid metabolism among protozoans and mammalian systems. However, these similarities also indicate that despite their more sophisticated lipid synthesis abilities, mammalian cells also require the uptake of exogenous lipids. It is surprising that none of the four giardial flippases are predicted to be localized in the ER, given that phospholipids, including sphingomyelin (Hernandez et al. 2007), are likely synthesized in the ER, thereby creating a need for transport molecules to move newly synthesized lipids through the ER membrane and ultimately to their designated location within the cell. Since PGPS is predicted to localize to the ER as well, it is logical to assume that newly generated PGs and sphingolipids would require a transport protein in order to pass through the ER membrane. In fact, it has been shown that flippases in other organisms, such as *Saccharomyces cerevisiae*, do indeed localize to the ER and are responsible for the translocation of not only phospholipids but also dolichol-phosphate-anchored oligosaccharides across the ER membrane (Sanyal et al. 2008). It is possible, however, that an undescribed lipid transport system exists solely for the purpose of transport out of organelles.

Table 4. Predicted open reading frames and Pfam matches of giardial PGPS, PSD, and Flippases.[a]

Designation (gORF no.)	GenBank Accession	Match to Pfam (motif location)	Species with best BLASTP match (E-value)	Pfam family match
Flippase IA 16958	XP_001704967	E-value 3.1e-10 (aa 87-173)	*Pediculus humanis corporis* (2e-94) *Entamoeba histolytica* (2e-90)	E1-E2 ATPase
Flippase IA 10019	XP_001710085	E-value 5.3e-11 (aa 97-185)	*Homo sapiens* (5e-76)	E1-E2 ATPase
Flippase IIB 101810	XP_001707954	E-value 3.2e-8 (aa 207-301)	*Homo sapiens* (5e-26)	E1-E2 ATPase
Flippase IIB 38104	XP_001704293.1	E-value 1.2e-13 (aa 100-295)	*Rattus norvegicus* (4e-94)	E1-E2 ATPase
PGPS (7259)	XP_769290	2.3E-29 (aa 63-191)	*Opitutacaea bacterium* (1e-32) *Trichomonas vaginalis* (5e-25)	CDP-alcohol phosphatidyl transferase
PSD (16495)	XP_779868	1.1E-13 (aa 186-409)	*Plasmodium vivax* (2e-22)	Phosphatidylserine decarboxylase

[a]Putative genes encoding flippases, phosphatidylglycerolphosphate synthase (PGPS) and phosphatidylserine decarboxylase (PSD) were identified in *Giardia* using the NCBI and Protein Family (Pfam) database. Adapted from Yichoy et al (2009).

Table 5. Topology and localization predictions of giardial lipid metabolic enzymes using PsortII (Horton and Nakai 1997; Nakai and Horton 1999).

		Predictions		
Designation (gORF)	**GenBank Accession**	**Topology (Transmembrane domains)**	***k*-NN**	**Palmitoylation Sites**
Flippase IA (16958)	XP_001704967	9	plasma membrane 69.6%	12
Flippase IA (10019)	XP_001710085	10	plasma membrane 65.2%	7
Flippase IIB (101810)	XP_001707954	10	plasma membrane 78.3%	7
Flippase IIB (38104)	XP_001704293.1	10	plasma membrane 69.6%	9
PGPS (7259)	XP_769290	4	endoplasmic reticulum 43.5% plasma membrane 21.7% mitochondrial 21.7%	1
PSD (16495)	XP_779868	1	mitochondrial 60.9% cytoplasmic 26.1%	1

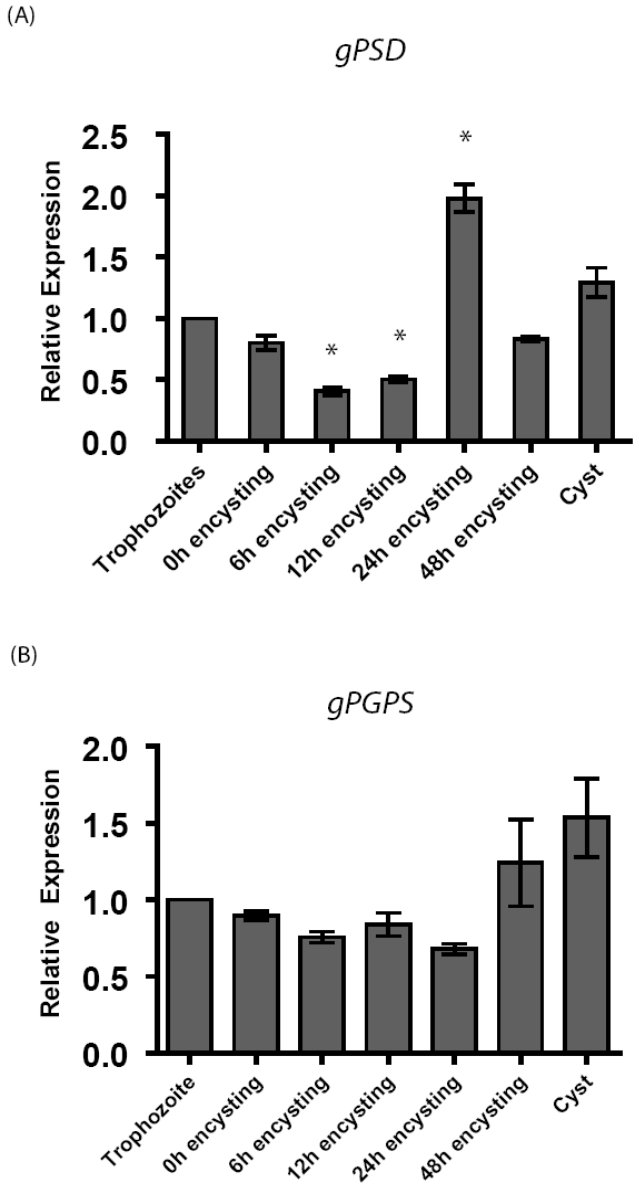


Figure 11. Differential expression of giardial phosphatidylserine decarboxylase (*gpsd*) and giardial phosphatidylglycerolphosphate synthase (*gpgps*) genes, relative to vegetative trophozoites. Experiments were carried out three times with three replicates each time. The data are the means and standard deviations of these replicates. An asterisk indicates significant difference compared to vegetative trophozoites with $p<0.05$. Panel A indicates the expression of *gpsd*, and Panel B denotes *gpgps* expression (Yichoy et al. 2009).

Chapter 4: Elucidating the mechanism of phosphatidylglycerol (PG) synthesis

Because phosphatidylglycerol (PG) is one of the two phospholipids newly synthesized by *Giardia* and abundant in trophozoites and cysts, it is likely that it's synthesis important for the growth and viability of this waterborne pathogen. This statement can be further supported by the fact that knocking down the *gpgps* gene (linked to PG synthesis) kills *Giardia* instantly (not shown). Genomic analyses (see Chapter 3) indicate that a majority of PG synthesis genes are not present in *Giardia*, and therefore that the question of how new PG is synthesized is extremely important. The goal of Chapter 4, therefore, is to delineate the mechanism of phosphatidylcholine (PC) internalization (because PC is the major phospholipid in the growth medium) and its subsequent conversion into PG and other phospholipids through remodeling reactions. Using fluorescently conjugated phospholipids, I show that the flipasse-like sulphohydryl (-SH) group containing transport proteins is involved in PC uptake across the plasma membranes of *Giardia*. These transporters are also efficient in transporting other amino-phospholipid (i.e., PE) but not glycerol-containing phospholipids such as PG. I speculate that PC is the major source of PG and that it most likely occurs by way of a headgroup remodeling step through an enzyme that is yet to be identified and characterized.

4.1. Materials and Methods

4.1.1. Materials

Stock solutions (100 mM) of N-ethylmalemide (NEM) and Verapamil (Sigma, St. Louis, MO) were prepared freshly before carrying out the experiments. Stock solutions of BODIPY-PE,

BODIPY-PC (Invitrogen, Carlsbad, CA), and NBD-PG (Avanti Polar Lipids, Alabaster, AL) were prepared by resuspending the lyophilized dyes in 1ml ethanol.

4.1.2. Labeling with the non-radioactive isotope [^{13}C]-glycerol

Media from confluent 8-ml culture tubes were discarded, and tubes were refilled with phosphate-buffered saline (PBS) supplemented with 2 mM cysteine and 100 µL dialyzed fetal bovine serum (FBS) per 8-ml tube, pH 7.1. [^{13}C]-glycerol was added directly to the culture tubes to create a final concentration of 5 mM, which was incubated at 37 ºC for 5 h. N-ethylmalemide (NEM) and Verapamil (flippase inhibitors) were also added to final concentrations of 200 µM and 50 µM, respectively (Biagini et al. 2000; Reiner et al. 2003). All labeling reactions were carried out in duplicate, and cells from duplicate tubes were later combined in order to achieve a sufficient number of cells to carry out the lipid isolation and analyses. Following the incubation period, liquid was removed by decanting the medium and refilling the tubes with PBS. Tubes were kept in ice-cold water for 20–30 min to remove the attached cells, and labeled cells were then harvested by centrifugation at 3,000 rpm for 7 min at 4°C to collect the cell pellets. The pellets were washed with PBS and kept at -20^{o}C until lipid extraction. Total lipids were isolated later by the Folch method, and phospholipids were fractionated using a Silica60 column, as previously described in Chapter 2.

4.1.3. Phospholipid analysis by linear ion trap mass spectrometry

MS spectra for fractionated lipids were acquired in a linear ion trap (LTQ XL, Thermo Fisher Scientific). Samples were dissolved in $CHCl_3$: CH_3OH: formic acid: NH_4OH (1:1:0.1%:0.1%) and were analyzed in negative-ion mode. Samples were injected using a Triversa Nanomate System (Advion), full-scan spectra were collected in the 550–900 *m/z* range, and MS/MS spectra were automatically collected by total-ion mapping using pulsed Q dissociation. The collision energy was set at 29% and Q = 0.7 for 0.1 ms, and helium was used as the collision gas. For quantitative analysis, samples were normalized to 5,000 cells/µl and spiked with 0.5 µM C12:0/C12:0–PG as an internal standard.

4.1.4. Labeling cells with a lipid-staining reagent.

Growth medium was discarded from culture tubes and refilled with PBS. Trophozoites were pre-incubated with 50 µM Verapamil or 200 µM NEM for 1 h at 37 °C. Following the pre-incubation, Nile Red, a lipid-staining reagent (Sigma, St. Lous, MO) was added to a final concentration of 10 µg/ml, incubated for 15 min, washed five times with PBS, and then harvested by centrifugation as described above. Trophozoites were then transferred to 4-well chambered slides (Nalgene Nunc, Naperville, IL) and allowed to attach for 30 min at 37 °C. Slides were then washed once in Coplin jars and trophozoites were fixed with 4% paraformaldehyde in 1x PBS for 15 min, washed again 3 times with 1x PBS and then allowed to air dry. Cover slips were mounted using DAKO Fluorescence Mounting Medium (DAKO

Corporation, Carpinteria, CA) and allowed to set overnight at 4 °C before analyzing by confocal microscopy.

4.1.5. Labeling with fluorescent lipid probes

The media in the culture tubes were discarded, and the tubes were refilled with PBS. Then the flippase inhibitors NEM and Verapamil were added, at the concentrations described above. Cells were pre-incubated for 1 h for Verapamil-treated cells and 45 min for NEM-treated cells. After the pre-incubation, the fluorescent lipid probes were added directly to the cultures in the following concentrations: BODIPY-PE, 200 μM; BODIPY-PC, 200 μM; and NBD-PG, 5 μM. Trophozoites were incubated with the fluorescent probes for 1 h at 37 °C, washed once, harvested by centrifugation as described above, and re-suspended in 1 ml PBS. Trophozoites were transferred to 4-well chambered slides (Nalgene Nunc, Naperville, IL) and incubated at 37°C for 30 min for Verapamil-treated cells and 45 min for NEM-treated cells to allow cells to attach to the slides. After the attachment period, slides were washed, fixed, and mounted as described above.

4.1.6. Analysis of lipid uptake by confocal microscopy

Images of fluorescent-labeled trophozoites were taken using an LSM5 Pascal laser-scanning, confocal microscope (Zeiss). For Nile Red BODIPY-PC and NBD-PG uptake experiments, images were scanned 16 times at a scan speed of 6. For BODIPY-PE, images were scanned 8 times, and a scan speed of 6 was used. Nine images were taken for each treatment

group, and control and fluorescence intensity were analyzed using LSM5 Pascal software, version 3.2 (Zeiss). To compare differences between treatments and controls in NBD-PG-labeled cells, one-way ANOVA with Dunnet's post test was performed. For all other fluorescent-labeling experiments, an unpaired, two-tailed t-test was performed. All statistical analyses included a sample size of at least n = 9 and a confidence interval of 95%, and were performed using GraphPad Prism version 4.00 for Windows® (GraphPad Software, San Diego, CA).

4.2. Results

4.2.1. Labeling with [^{13}C]-glycerol

The previous experiment from our laboratory suggested that [^{14}C]-labeled glycerol incorporates into PG in *Giardia* (unpublished). However, these studies were performed using TLC, making it difficult to identify specifically the species into which the radio-labeled bases were incorporating. Therefore, these assays were repeated using [^{13}C]-glycerol, a non-radioactive isotope, which allowed for analysis by mass spectrometry. Because bases with a two-carbon label were used (Figure 12), a 2-Dalton shift should be evident in the MS/MS spectrum if the isotopes are incorporated,. Therefore, if a [^{13}C] 1, 3-Glycerol C_2 label incorporates into a PG, a 2-Dalton shift for *m/z* 153 to *m/z* 155 (Figure 13) should be seen with the peak corresponding to the glycerol phosphate moiety of PG. While a few technical problems caused a limited amount of labeling, these studies reveal that [^{13}C]-glycerol incorporates into the three most abundant PGs (Yichoy et al. 2009): C16:0/C16:0-PG, C18:0/C14:0-PG, and C18:0/C16:0-PG (Figure 13). Labeling with non-radioactive glycerol was also performed in the presence of NEM, a flippase inhibitor, and Verapamil to determine whether remodeling of PLs is dependent upon the uptake

of exogenous head groups. Because low levels of incorporation were observed, treatment with these drugs did not result in significant differences in incorporation levels. Interesting results were observed in the full-scan spectra, however (Figure 14). In NEM-treated cells, the total amount of PGs appears to be dramatically decreased. This indicates that *Giardia* is dependent upon lipid transport systems in order to generate new PLs. Differences in lipid composition were not observed between untreated trophozoites and Verapamil-treated cells, which suggests that the flippase-like transporters present in this protozoan are not Ca^{2+} dependent.

4.2.2. Uptake and incorporation of fluorescent lipid probes

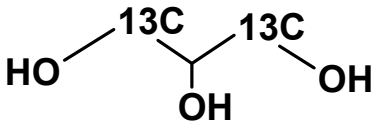
To further examine whether the distribution and uptake of lipids is dependent upon a PL transport system, trophozoites were treated with NEM and Verapamil and then labeled with the lipid tracker Nile Red, as well as with the fluorescent lipid probes BODIPY-PC, BODIPY-PC, and NBD-PG. Major differences were not observed in overall lipid distribution when cells were labeled with Nile Red (Figure 15). However, localization of lipids in the nuclear region appeared to be decreased in both NEM-treated cells, but increases when cells are treated with Verapamil. This indicates that while uptake of other lipids (ie; cholesterol and sphingolipids), is not dependent upon a flippase-like enzyme, transport between organelles is indeed affected. Also, while Verapamil and NEM are considered broad-spectrum inhibitors, this data indicates that formation of clathrin-coated vesicles, which is responsible for the uptake of ceramide, is not affected by either of these compounds (Hernandez et al. 2007).

More marked differences were observed in BODIPY-PC and -PE uptake and incorporation in the presence of flippase inhibitors (Figures 16 and 17). In fact, both BODIPY-

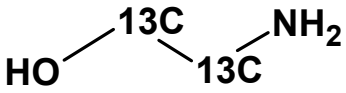
PC and -PE uptake are dramatically decreased, and the fluorescence intensity is many times lower than untreated controls. For Verapamil-treated cells, the only observed difference is decreased localization of BODIPY-PE in the membranes, but there is no difference for BODIPY-PC distribution. For cells labeled with NBD-PG (Figure 18), while fluorescence intensity is decreased with both inhibitors, the difference is not quite as dramatic as with BODIPY-PC and -PE, indicating that the flippase-like transporter localized in the giardial plasma membrane is specific to PC and PE and that PG can be internalized via other mechanisms. Although uptake of NBD-PG is significantly affected, the fact that it is not completely reduced indicates that PG can be internalized from the host through another transport mechanism.

Conclusions

The [^{13}C]-incorporation and fluorescent uptake assays described in this chapter raised several possibilities regarding lipid scavenging and PG synthesis by *Giardia*. The confocal analysis reveals that *Giardia* is largely dependent upon PC from the host, and most likely PG can be generated by the parasite itself. Therefore, it can be concluded that while *Giardia* has more lipid-synthesis activities than previously thought, this organism is largely dependent upon exogenous sources of lipids, particularly PC.



Glycerol-1,3-$^{13}C_2$



Ethanolamine-$^{13}C_2$

Figure 12. Structures of non-radioactive isotopic phospholipid precursors.

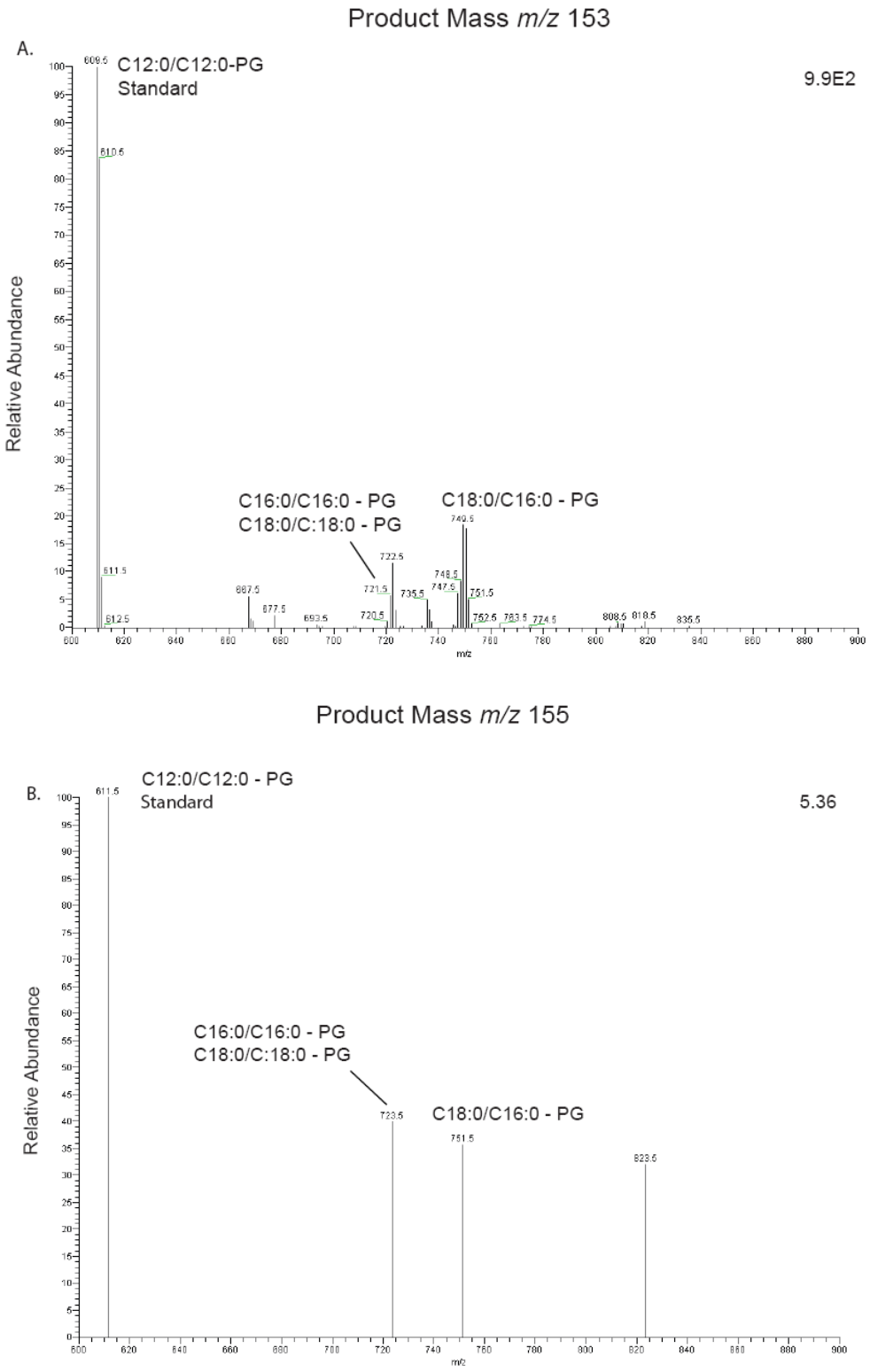


Figure 13. Product mass spectra for [^{13}C]- glycerol incorporation.

A. Product mass *m/z* 153. B. Product mass *m/z* 155.

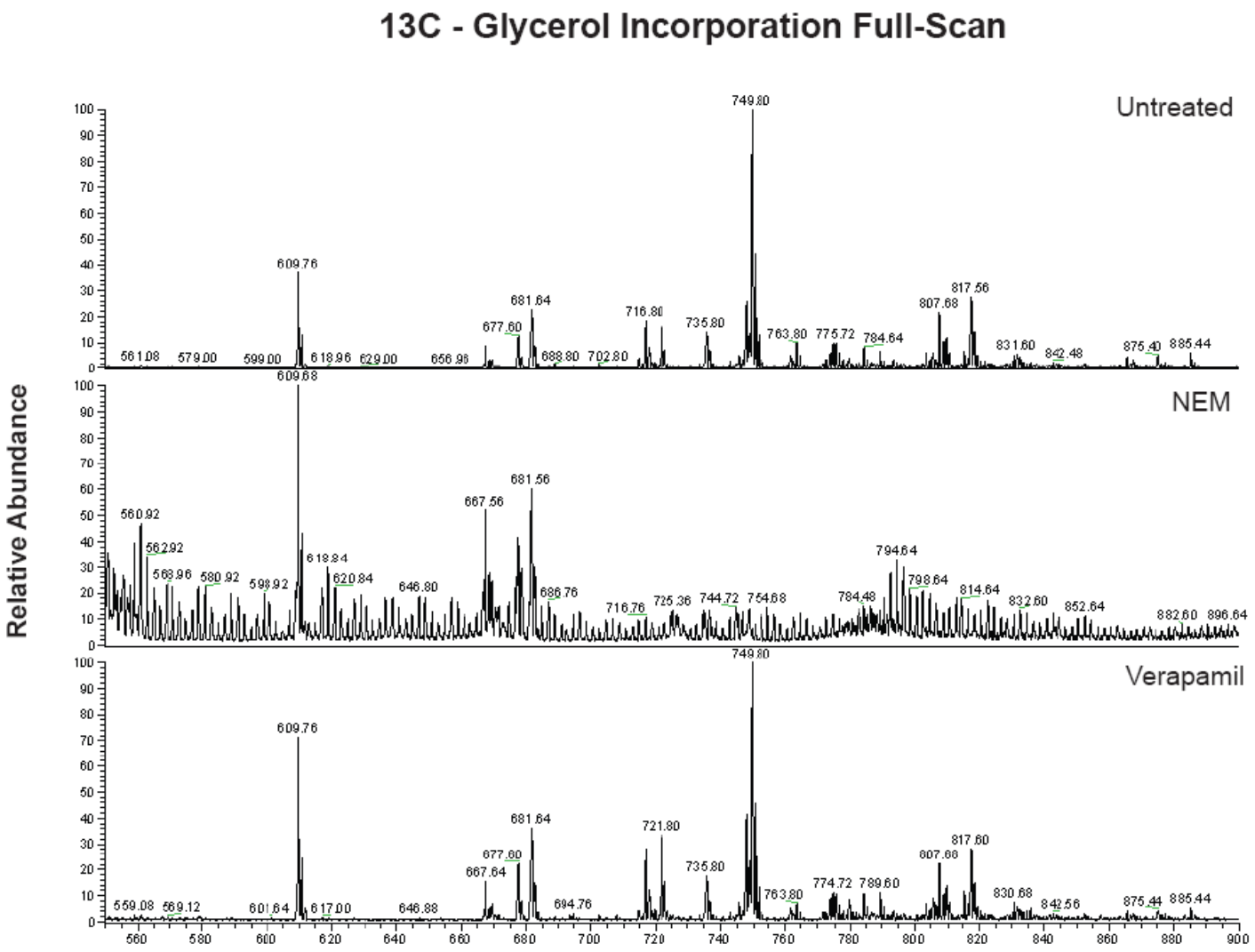


Figure 14. Full-scan spectra of [^{13}C] – glycerol incorporation. The internal standard and its adducts are represented by peaks at *m/z* 609, 667, 677 and 681.

Figure 15A.

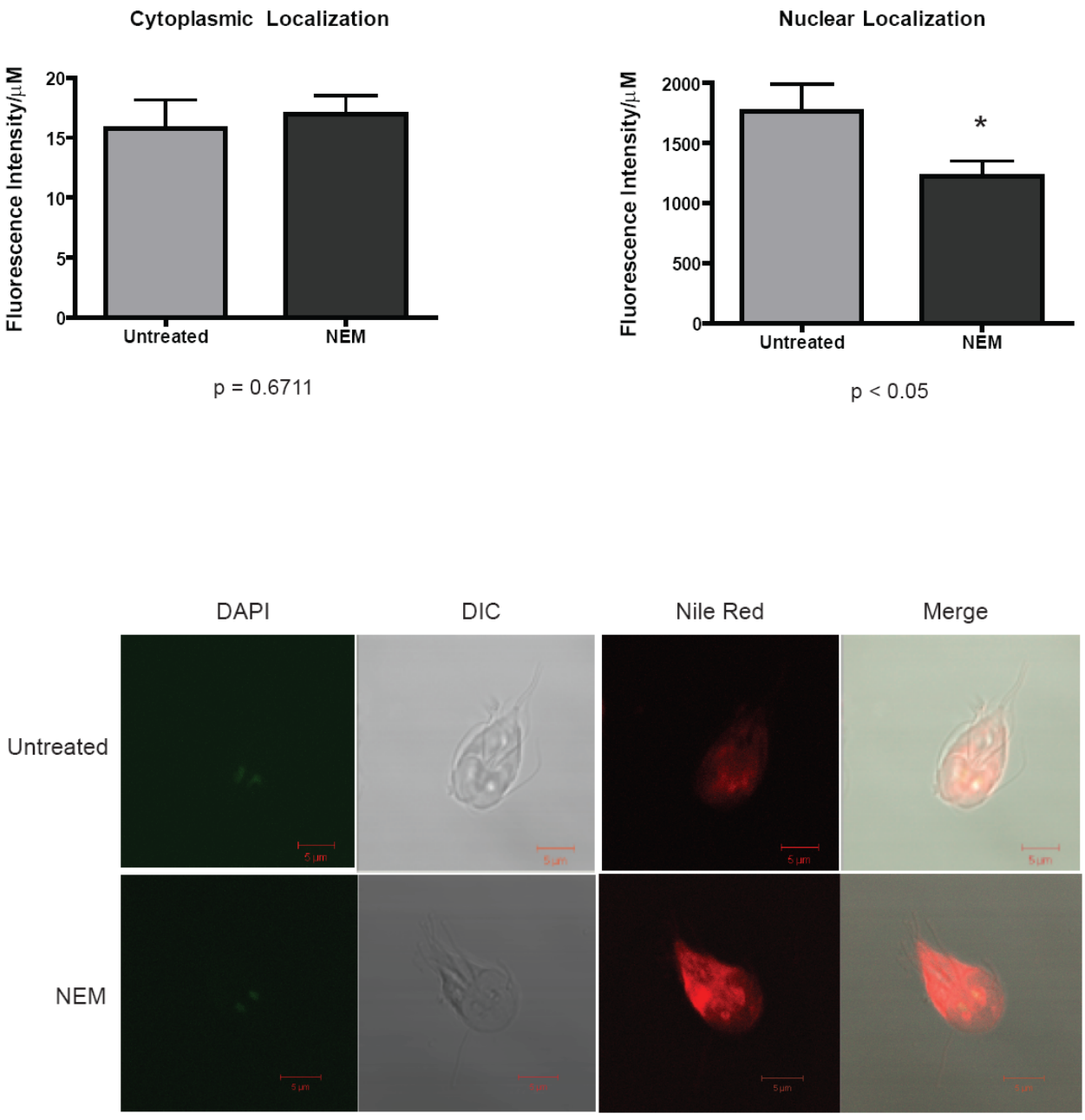

Cytoplasmic Localization
Fluorescence Intensity/μM
20
15
10
5
0
Untreated
NEM
p = 0.6711
Nuclear Localization
Fluorescence Intensity/μM
2000
1500
1000
500
0
*
Untreated
NEM
p < 0.05
DAPI
DIC
Nile Red
Merge
Untreated
NEM

Figure 15B.

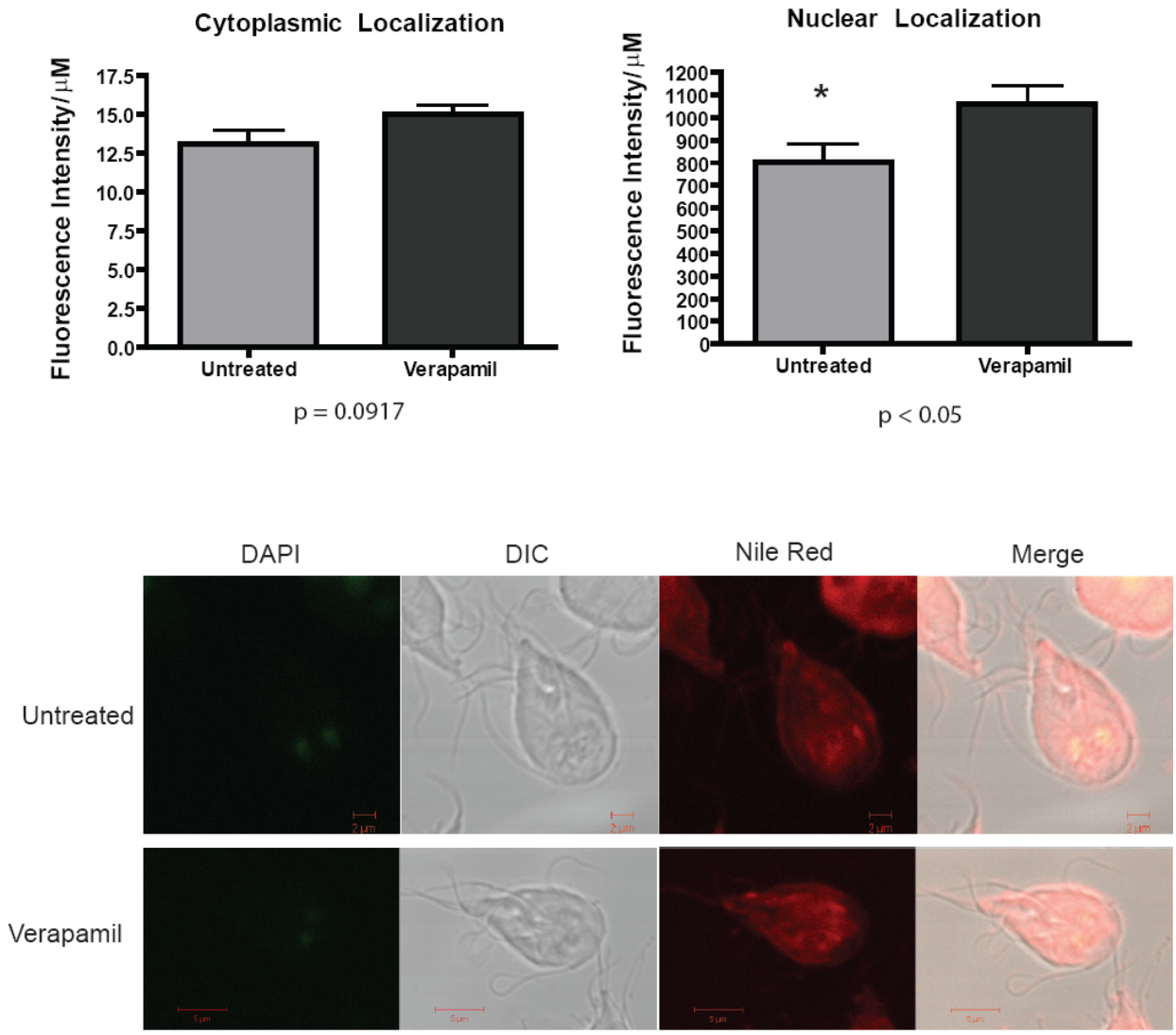


Figure 15. Uptake and incorporation of Nile Red. Confocal images showing distribution and statistical analysis of quantitation of fluorescence intensity/µM. P-values for each experiment are shown below each graph. A. Nile Red incorporation in the presence of NEM. B. Incorporation in Verapamil-treated cells.

Figure 16A.

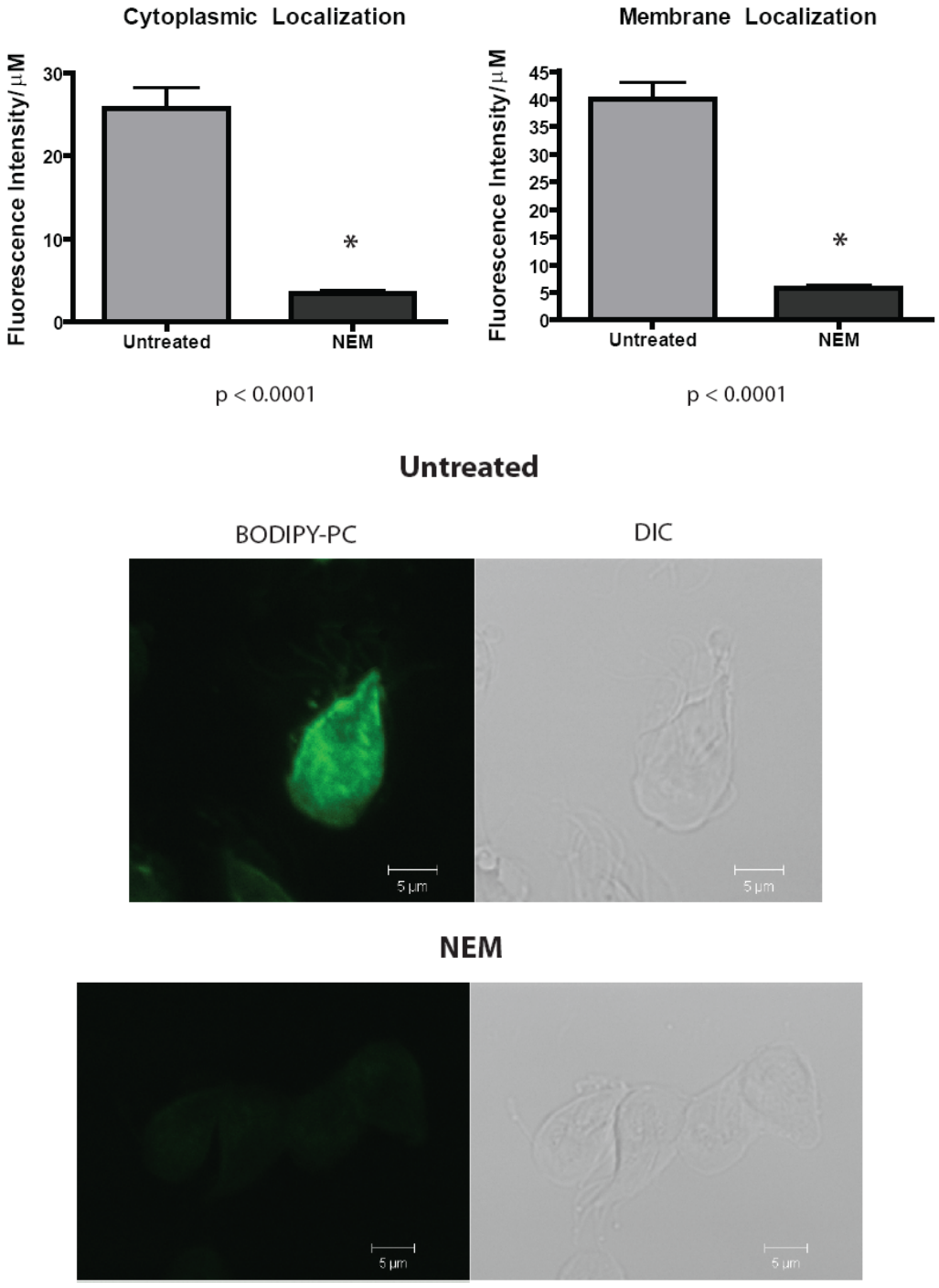

Cytoplasmic Localization
Fluorescence Intensity/μM
30
20
10
0
*
Untreated
NEM
p < 0.0001
Membrane Localization
Fluorescence Intensity/μM
45
40
35
30
25
20
15
10
5
0
*
Untreated
NEM
p < 0.0001
Untreated
BODIPY-PC
DIC
5 μm
5 μm
NEM
5 μm
5 μm

Figure 16B.

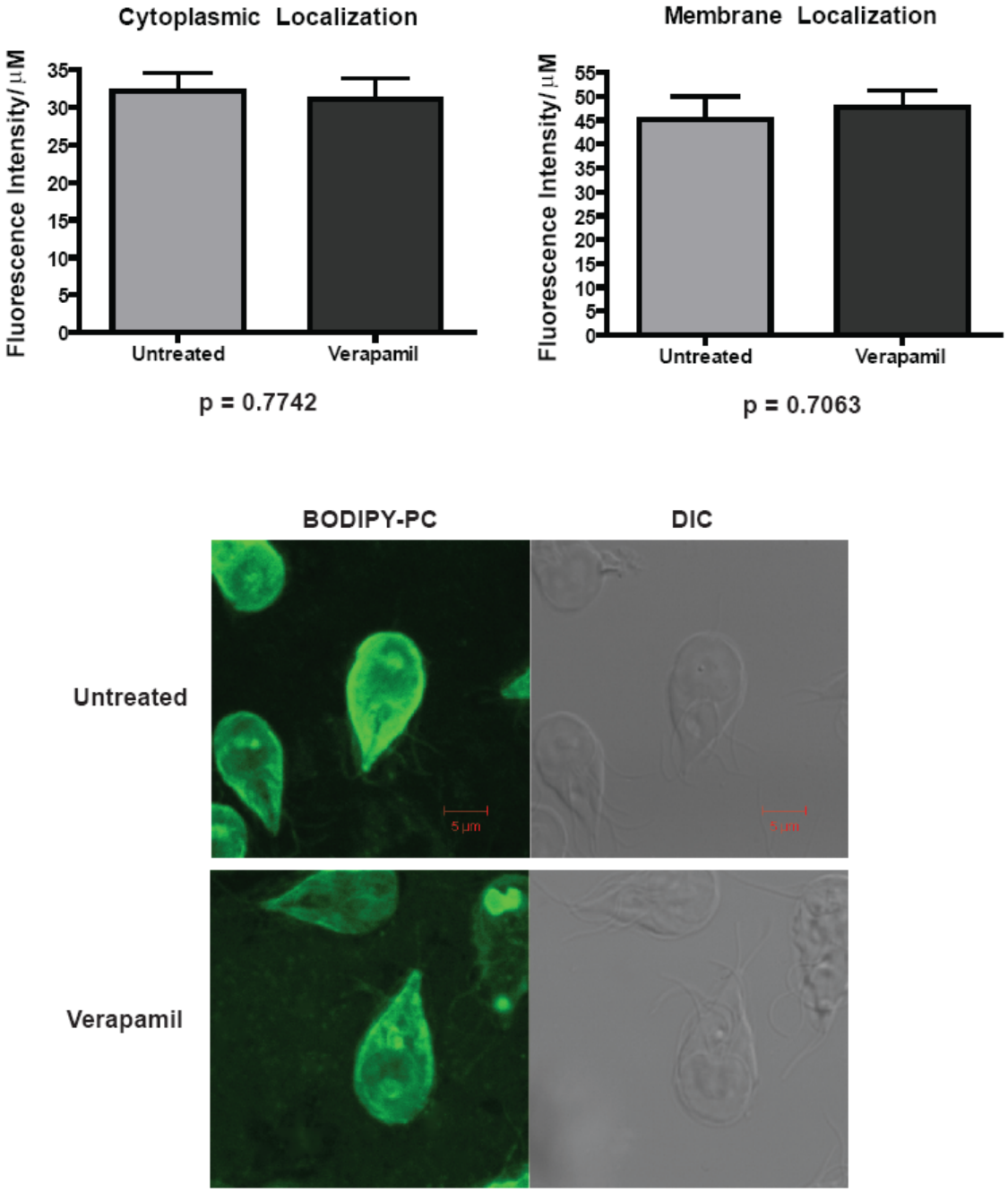
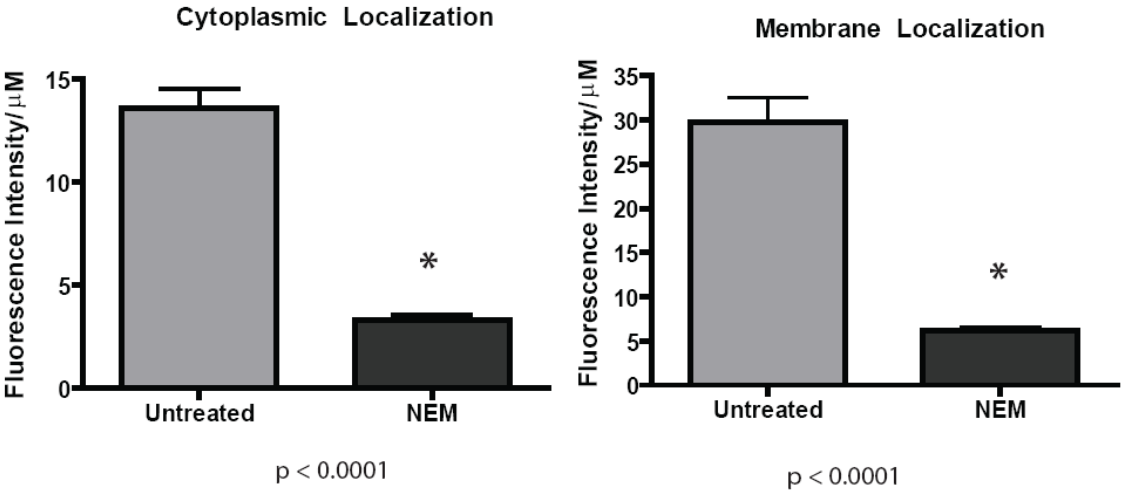


Figure 16. Uptake and incorporation of BODIPY-PC. Confocal images showing distribution and statistical analysis of quantitation of fluorescence intensity/µM. P-values for each experiment are shown below each graph. A. NEM-treated cells. B. Verapamil-treated cells.

Figure 17A.


Cytoplasmic Localization
Fluorescence Intensity/μM
15
10
5
0
*
Untreated
NEM
p < 0.0001
Membrane Localization
Fluorescence Intensity/μM
35
30
25
20
15
10
5
0
*
Untreated
NEM
p < 0.0001

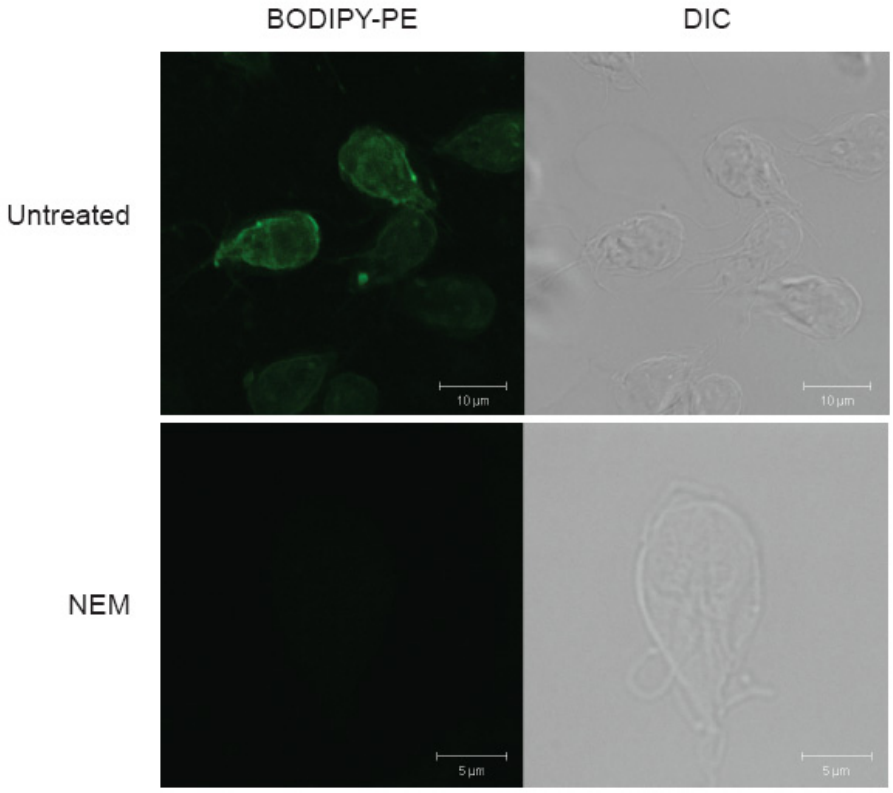

BODIPY-PE
DIC
Untreated
NEM
10 μm
10 μm
5 μm
5 μm

Figure 17B.

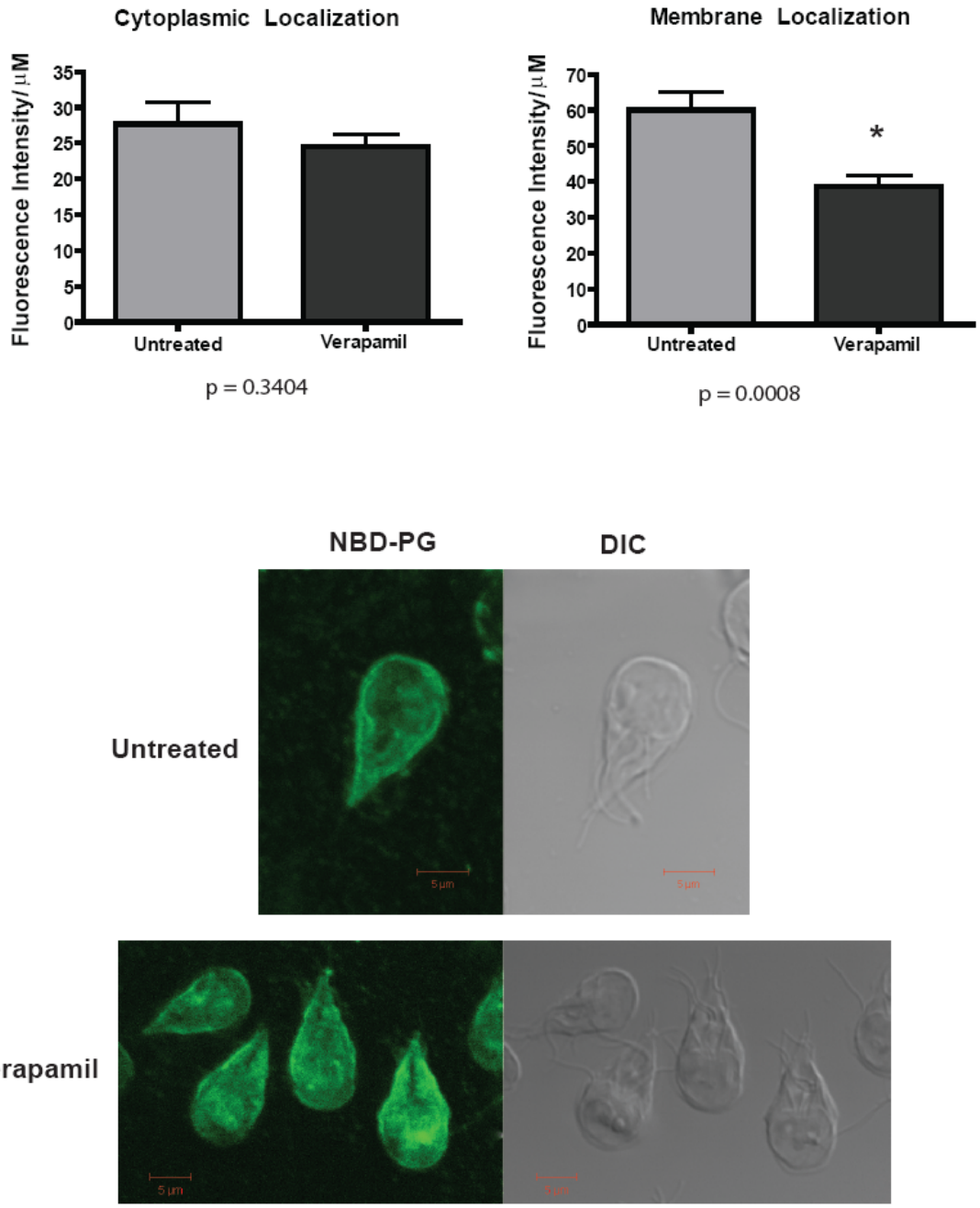
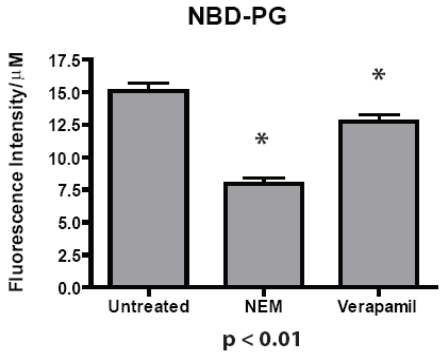


Figure 17. Uptake and incorporation of BODIPY-PE. Confocal images showing distribution and statistical analysis of quantitation of fluorescence intensity/µM. P-values for each experiment are shown below each graph. A. NEM-treated cells. B. Verapamil-treated cells.

A.



B.

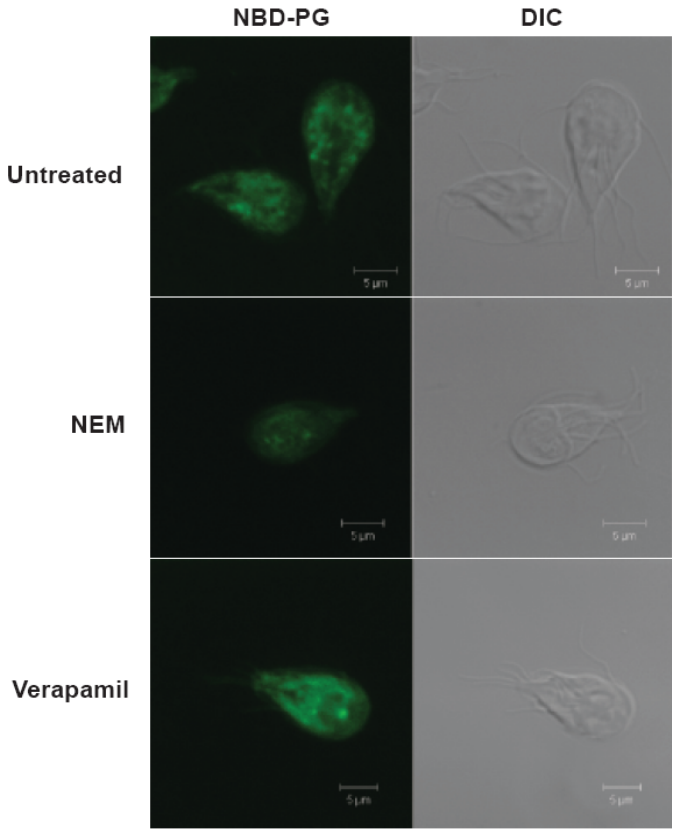


Figure 18. Uptake and incorporation of NBD-PG. A. statistical analysis of quantitation of fluorescence intensity/µM. P-values for each experiment are shown below each graph. B. Confocal images showing distribution of NBD-PG.

Chapter 5: Discussion and Future Directions

Because of its inability to synthesize membrane lipids *de novo*, it has been proposed that *Giardia* has evolved well-orchestrated mechanisms to import and utilize pre-formed lipids and fatty acids from the cell-exterior (Gibson et al. 1999; Subramanian et al. 2000; Das et al. 2001). Many of these exogenous lipids/fatty acids undergo remodeling before they are incorporated into giardial membranes (Subramanian et al. 2000; Das et al. 2002). Although, these reports provide some insights into the metabolism of exogenously acquired lipids, the detailed mechanisms of lipid metabolism in *Giardia* are yet to be delineated.

The current study was undertaken to address two fundamental questions related to the lipid metabolism by *Giardia*. First, we asked whether giardial lipid compositions are similar to that of the culture medium, as proposed by other investigators (Jarroll et al. 1981; Kaneda and Goutsu 1988; Mohareb et al. 1991; Ellis et al. 1996), and second we asked whether any new membrane lipids are synthesized by this parasite.

We began our experiments by identifying membrane lipids and fatty-acid constituents through ESI-QTOF-MS. Lipid samples from trophozoites, encysting cells, and cysts were analyzed in positive- as well as negative-ion mode to retrieve overall information. Interestingly, significant differences in the lipid species present in any stage of the life cycle were not detected (Fig. 5). Furthermore, our results indicate that PG, PE, and PC are major phospholipids in *Giardia* and most likely synthesized *de novo*, since the analysis of phospholipid content in bovine bile and serum yielded no PG or PE (Tables 1 and 2, and Fig. 7). In another experiment (not shown), we examined phospholipid compositions of the culture medium (containing peptone, yeast extract, salts, serum, and bile) by thin-layer chromatography, and again, no PG

and PE could be identified. Although PG is abundant in bacteria, trace amounts of this acidic PL can also be found in mammalian and protozoan cells (Besteiro et al. 2008). On the other hand, PE is present in both prokaryotic and eukaryotic membranes and is involved in the formation, fusion, and vesiculation of lipid bilayers (Besteiro et al. 2008). Various biochemical and structural studies (Besteiro et al. 2008; Zhao et al. 2008) have indicated that PG interacts with other charged lipids (i.e., PC and PE) and that it acts as a membrane stabilizer in addition to regulating the lipid-protein interactions in bacterial membranes. PG-PE interactions are also important for vesicle morphology, membrane permeability, and overall maintenance of the membrane stability (Garidel and Blume 2000). Based on these studies, and considering the challenges that *Giardia* meet in the human gut, it can be proposed that the interactions between PG and PE as well as PG and PC are important for this protozoan parasite to survive and complete its life cycle in the hostile environment of the human small intestine. Furthermore, the abundance of PG in *Giardia* could also be traced back to its evolutionary origin. It has been demonstrated that *Giardia* synthesizes a cryptic mitochondrion or mitosome as part of its putative mitochondrial ancestry (Tovar et al. 2003; Regoes et al. 2005). A recent report suggests that cardiolipin (a PG dimer), which is present in bacterial membrane, is also present in hydrogenosome (i.e., a mitosome-like organelle) in *Tritrichomonas foetus* (de Andrade Rosa et al. 2006). Therefore, it can be hypothesized that the abundance PG in *Giardia* could be a signature of its endosymbiotic past and that it originated from alphaproteobacteria during symbiosis (van der Giezen and Tovar 2005). However, more in-depth experiments are required to support this notion.

It appears that both PG and PE in *Giardia* are generated by base-exchange reactions rather than the *de novo* (CDP-DAG) synthesis. This postulation can further be supported by the

fact that when live trophozoites were metabolically labeled with radioactive glucose, threonine, glycerol, and acetate no radioactivity could be detected in lipid fractions (Jarroll et al. 1981). Conversely, we found that radiolabeled bases, fatty acids, and other preformed lipids can be assembled to larger lipid molecules (Das et al. 2002). In a separate experiment, it was noted that [^{14}C]-glycerol and [^{14}C]-ethanolamine are incorporated into PG and PE, which suggests that *Giardia* might synthesize enzymes that allows these bases (i.e., glycerol and ethanolamine) to incorporate them into respective phospholipids. Intriguingly, we could not detect PS in *Giardia*, bile, or serum. This could be due to the reasons that PS is a minor PL that exist in few molecular species. It is possible that during our analyses, PS was broken down to serine and diacylglycerol. In fact, Table 2 shows the presence of DAG when phospholipid composition of bile and serum was determined by MS analysis. Nonetheless, when live trophozoites are cultured in the presence of [^{14}C]-serine the majority of serine is assimilated into PE, which indicates the presence of strong PSD activity and may explain why we failed to detect PS in *Giardia* (Das 2005).

As has previously been demonstrated (Kaneda and Goutsu 1988; Mohareb et al. 1991; Ellis et al. 1996) and also confirmed by our MS analysis (Table 1, Fig. 6), PC is also abundant in *Giardia* and is the only major PL available in the growth medium. In light of the fact that the medium contains only PC and *lyso*-PC, the number of phospholipid species (i.e., PG, PE, and PI) identified in trophozoite and cyst supports the idea that base-exchange enzymes are present in this organism (Subramanian et al. 2000). However, except for *gpsd*, no genes for base-exchange enzymes have so far been revealed from the genome project, and therefore detailed biochemical analyses are required for further characterization of these enzymes.

Our GC-MS analysis of total fatty acid content reveals that *Giardia* contains few additional fatty acids that are not present in the medium. Fatty acids like 21:1, 23:0, 24:0, and

24:1 are only present in *Giardia*, which suggests that these are newly generated fatty acids produced by elongation and desaturation reactions (Fig. 8, Table 3). The presence of a fatty acid desaturase enzyme has already been reported by Ellis et al. (1996), and the *Giardia* Genome Database has identified the presence of a fatty acid elongase gene; the two could be responsible for the exchange and elongation of acyl chains, respectively. As to the presence of odd-carbon fatty acids (OCFAs), in our GC-MS analysis conditions we could not determine whether they were linear or methyl-branched structures.

Our genomic and *in silico* analyses further emphasize that lipid metabolism is crucial to parasite survival. The fact that *pgps* and *psd* are continually transcribed throughout the life cycle suggests that both of these the sequences for both enzymes are housekeeping genes. Furthermore, unpublished qRT-PCR data generated in our laboratory show that all four flippase genes are transcribed throughout the life cycle and that, in fact, one is expressed five-fold higher in pre-encysting cells than in trophozoites (Aguilera et al., 2009, unpublished). This indicates that lipid uptake continually occurs during the encystation period, especially while the cell is building energy stores, constructing the cyst wall, and preparing for its semi-dormant state.

In addition to the lipidomic and transcriptional analysis, I have also used various bioinformatics tools to predict the cellular localization of PGPS, PSD, and the four flippases. It is surprising that none of the flippases are localized in the ER because lipid assembly and remodeling likely happen in this organelle. It would be logical for a lipid transporter to localize in the ER and Golgi, although it is possible that another uncharacterized transporter is present.

Our functional analyses confirm the fact that *Giardia* is dependent upon exogenous sources of lipids. The fact that the full-scan spectra from NEM-treated cells show only background levels of PG and PE emphasizes that, while *Giardia* is capable of some lipid

synthesis activities, this protozoan is still largely dependent upon a lipid source from the extracellular milieu, which may act as a sort of "template" from which new PLs can be constructed. Also, since the fluorescent labeling assays show very minimal uptake of BODIPY-PC and -PE, it can be concluded that the four Giardial flippase-like transporters are PC and PE specific, which suggests that some other PG-specific internalization system exists. Furthermore, while the [^{13}C]-glycerol and -ethanolamine labeling experiments require that the culturing conditions be perfected, the [^{13}C]-glycerol incorporation indicates that PL remodeling through exchange of phosphate head groups does indeed occur.

5.1. Final Conclusions

Based on biochemical data as well as available genomic and bioinformatics tools, I have constructed a model of phospholipid, fatty acid, and diacylglycerol DAG remodeling (Figure 19). PC and lyso-PC enter the cell by way of a flippase-like transporter with multiple trans-membrane domains. PC can be remodeled into PG through multiple though yet-to-be-characterized steps by PGPS. PG, in turn, can be used to form PI by a PI synthase in a base-exchange reaction. Conversely, PC may also be demethylated by a methyltransferase to form PE. If PE is used to generate PS, it is likely converted back to PE because PS was not evident in the lipidomic analysis. The lyso-PC taken up from the growth medium may also be remodeled into other PCs through the exchange of acyl groups. Quite likely, these same enzymes, lysophosphatidic acid acyltransferase and 1-acyl-sn-glycerol-3-phosphate acyl transferase, are also responsible for exchanging fatty acid chains to form remodeled DAG and triacylglycerols. Finally, elongation and desaturation of fatty acids also occurs.

The work presented here begins to elucidate the mechanisms of lipid uptake and remodeling in *Giardia*. In order to continue this endeavor, it would be of interest to confirm that PG is generated from PC by labeling with [^{14}C]-glycerol and chasing with non-radioactive PC. If PG is indeed generated from exogenous PC, PG levels should be higher in the samples exposed to cold PC. It would also be of interest to knock down each individual flippase in order to examine whether the level of any phospholipids is decreased in order to determine the specificity of each transporter. Furthermore, while the PSORTII predictions of subcellular localization are useful, tagging each flippase as well as PGPS and PSD with a GFP to localize these within the cell would provide more concrete data. Finally, the isolation and characterization of a PG-remodeling enzyme, as I have proposed in this dissertation, should be unique and useful for screening small molecules by high-throughput screening as part of an anti-giardial drug-discovery program currently ongoing in our laboratory.

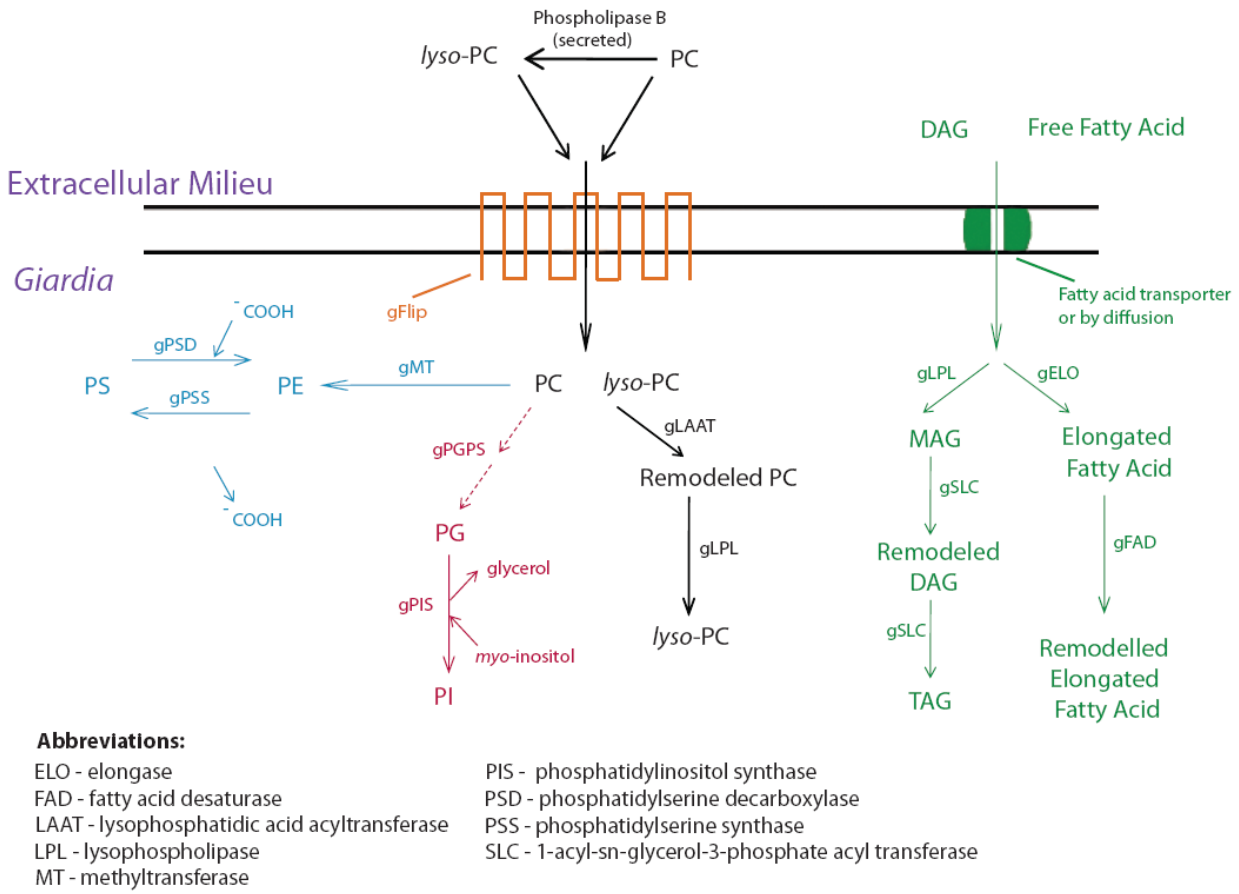


Figure 19. Model of phospholipid, fatty acid and diacylglycerol remodeling in *Giardia*.

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Appendix

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Thank you,

Mayte Yichoy
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Curriculum Vita

Mayte Yichoy was born on May 12, 1982 in Lima, Peru. The youngest of three children of Victor M. Yichoy and Irma Cavero, and raised by her grandparents, Victor and Julia Yichoy, she finished her undergraduate studies at Ripon College in 2004, obtaining the degree of Bachelor of Arts in Biology with a minor in Chemistry. In the fall of 2004 she enrolled at The University of Texas at El Paso and will graduate in the spring of 2009. During the five years spent at UTEP, she has been working under Drs. Siddhartha Das and Steve Aley on lipid uptake and metabolism in *Giardia lamblia*. She has presented her work at numerous conferences, including the Molecular Parasitology Meeting in Woods Hole, MA in 2008 and 2007 as well at the annual meeting of the Rio Grande Branch of the American Society of Microbiology held in Albuquerque, NM in 2008. In 2009 she published an article in Molecular and Biochemical Parasitology titled "Lipidomic analysis reveals that phosphatidylglycerol and phosphatidylethanolamine are newly generated phospholipids in an early-divergent protozoan, *Giardia lamblia*." She has also taught several courses at UTEP, including General Microbiology, Pathogenic Microbiology, and Microbial Physiology. In the Spring of 2009 she received the Dodson Graduate School Dissertation Fellowship from UTEP.

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