Bistability In The Protozoan Parasite *Giardia lamblia*

A dissertation presented by

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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology in the Graduate School of Science of Northeastern University, September 2012
ABSTRACT

*Giardia lamblia* is an important protozoan parasite, the transmission of which from one host to the other, and survival outside the host are dependent on differentiation of vegetative trophozoites into infectious cysts (encystment). Like many protozoa, *Giardia* trophozoites differentiate into cysts in response to nutrient deprivation or other unfavorable environmental changes. *Giardia*, a lipid auxotroph, encysts when bile sequesters lipids that they require (Jarroll *et al.*, 1981, Luján *et al.*, 1996b). During encystment, *Giardia* trophozoites elicit a unique pattern of gene expression resulting in the synthesis and transport of cyst wall components ultimately resulting in the formation of a protective cyst wall.

Microorganisms that deal with constantly changing environmental conditions such as those experienced by *G. lamblia* in the host’s intestinal tract have evolved additional strategies to increase their chances of survival. Here, evidence is presented that *Giardia* populations, in addition to forming cysts, employ bistability as a means of survival. Trophozoite populations respond heterogeneously to both encystment and vegetative growth; induction of encystment results in a bistable outcome where a proportion of the population encyst while a significant proportion remains undifferentiated thus producing a phenotypically heterogeneous population, and in the absence of exogenous bile in vegetative cultures a small, but significant, proportion of the population form cysts. The phenotypic heterogeneity
observed prepares *G. lamblia* populations for survival in both favorable and unfavorable environments.

Even though heterogeneity ensures that the different members of *Giardia*’s populations contribute to the survival of the population as a whole, there was no evidence that the sub-populations produced during encystment promote the heterogeneity observed; encysting and non-encysting trophozoites (within encysting cultures) neither exclusively promoted nor inhibited encystment in freshly induced trophozoites. In both cases, high bile conditions were required to produce statistically significant encystment levels. The evidence presented however shows that in addition to high bile conditions, the regulatory pathways for encystment can be spontaneously activated to achieve the heterogeneity observed in the vegetative cultures. There is, therefore, an element of stochasticity involved. The data also suggest that extracellular signaling molecules are neither produced nor used during the induction of encystment, which explains the lack of influence on encystment that occurred when cyst/encysting trophozoites were co-cultured with fresh trophozoites.

Signaling pathways induced during encystment also exhibit bistable properties; trophozoites induced to form cysts become committed after a short period of time and the production of encystment specific proteins is stably maintained when encystment is interrupted. After 3-6 h in inducing conditions, encysting trophozoites continue to encyst regardless of whether the inducing stimulus
remains. Transfer of encysting cells into non-inducing conditions does not result in the interruption of encystment specific protein production, which suggests the involvement of regulatory pathways with the ability to “remember” a transient signal long after its removal. These observations suggest that the regulatory pathways induced during encystment exhibit hysteresis. This property enables encysting trophozoites to complete the encystment process should the triggering unfavorable condition(s) become sustained or prolonged. Bistability of the regulatory pathways induced during encystment also ensure that in situations where the presence of the inducing signal occurs only shortly, the induction process can be easily aborted to conserve energy and promote growth. Bistability appears to play an important role in Giardia’s success as a parasite.
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>Agm</td>
<td>Phospho N-acetylglucosamine mutase</td>
</tr>
<tr>
<td>ARID</td>
<td>AT-rich interaction domain</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
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<td>Cyst wall filament proteins</td>
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<td>CWP gene</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>Differential interference contrast microscopy</td>
</tr>
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<td>Deoxyribonucleic acid</td>
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<td>Enzyme commission</td>
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<td>ER</td>
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</tr>
<tr>
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<td>Extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>ESVs</td>
<td>Encystment specific vesicles</td>
</tr>
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</tr>
<tr>
<td>FITC</td>
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<td>GalNAc</td>
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<td>Glucosamine-6-phosphate</td>
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<tr>
<td>gnp</td>
<td>GlcN-6-P-deaminase</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>nBLAST</td>
<td>Nucleotide BLAST</td>
</tr>
<tr>
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<td>No bile medium</td>
</tr>
<tr>
<td>NET</td>
<td>Non-encysting trophozoites</td>
</tr>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<td>q-PCR</td>
<td>Quantitative PCR</td>
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<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SREBPs</td>
<td>Sterol regulatory element binding-proteins</td>
</tr>
<tr>
<td>Uae</td>
<td>UDP-N-acetylglucosamine 4'-epimerase</td>
</tr>
<tr>
<td>Uap</td>
<td>UDP-GlcNAc pyrophosphorylase</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine 5'-diphosphate</td>
</tr>
<tr>
<td>UDP-GalNAc</td>
<td>UDP-N-acetylgalactosamine</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine 5'-triphosphate</td>
</tr>
<tr>
<td>VAD</td>
<td>Ventral adhesive disk</td>
</tr>
<tr>
<td>VSP</td>
<td>Variable surface proteins</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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CHAPTER 1: INTRODUCTION

*Giardia intestinalis* (syn. *G. duodenalis*, *G. lamblia*), a ubiquitous intestinal protozoan parasite, commonly causes infection (Giardiasis) in a wide range of hosts including rodents, livestocks, and humans (Erlandsen *et al.*, 1988; Rendtorff, 1954; Thompson, 2008). *Giardia* is a significant burden in humans, where it has been associated with acute epidemics and chronic illness, especially in children. The World Health Organization (WHO) estimates that there are about 500,000 new cases of giardiasis reported each year in developing countries and over 200 million people have giardiasis in these countries (Thompson, 2000). There is a lower but significant incidence of giardiasis in developed countries; there are about 20,000 cases of giardiasis reported annually in the USA (Thompson, 2008; Yoder and Beach, 2007). Giardiasis is the most common cause of waterborne outbreaks of diarrhea in the United States, and according to the U.S. Center for Disease Control and Prevention, it is the eighth most frequent cause of reportable infections in the U.S. (Barwick *et al.*, 2000). The rising incidence of giardiasis worldwide has led to its inclusion in the WHO’s Neglected Diseases Initiative (Savioli *et al.*, 2006).

*Giardia* is considered the most commonly isolated intestinal parasite throughout the world (Marshall *et al.*, 1997; Craun, 1990) and accounts for 17% (1.87 million) of global annual morbidity in children below the age five years, (Boschi-Pinto, 2008; Lawn *et al.*, 2006; Bryce *et al.*, 2005).

*Giardia* infection begins when viable cysts are consumed and its transmission from
one host to the other is very simple: it spreads by a direct fecal-oral route through consumption of contaminated water, food or fomites. This simple means of transmission makes *Giardia* infection an important concern in childcare centers, the nature of which can facilitate the spread of *Giardia*, and giardiasis happens to be most common in day-care centers (Kulda and Nohýnková, 1995). Unlike most bacterial infections, a large inoculum is not required to establish *Giardia* infection; as few as 10 viable *Giardia* cysts can cause giardiasis (Nash *et al*., 1987; Rendtorff, 1954) and infected persons may shed up to $10^9$ cysts in their stool per day (Rendtorff, 1954)

Infection with *Giardia* can be either asymptomatic or symptomatic manifesting as mild, acute or chronic giardiasis. Symptomatic giardiasis begins about 7-19 days after infection and is characterized by diarrhea, abdominal pain and flatulence. Patients with acute giardiasis may also experience fatigue, anorexia, nausea, steatorrhea, and fever (Kulda and Nohýnková, 1995; Farthing, 1994) and in cases where chronic giardiasis develops, patients experience persisting or recurring flatulence, abdominal distention, gastric cramps, loose stool, and general malaise (Farthing, 1994). Giardiasis is often associated with malnutrition and growth retardation especially in children (Fraser *et al*., 2000); most patients experience carbohydrate, fat, and micronutrient malabsorption; malabsorption of fat, lactose, vitamin A, and vitamin B12 have been reported in patients with chronic giardiasis, (Gardner and Hill, 2001; Farthing, 1994). The infection can last from three days to several months and the persistence may be continuous or sporadic (Gardner and
Drugs available for treating *Giardia* infection in humans include nitroimidazoles such as metronidazole (Flagyl®) and tinidazole (Tindamax®, Fasigyn®); the nitrofuran – furazolidone (Furoxone®); and the benzimidazole – albendazole. The nitroimidazoles (metronidazole and tinidazole) and albendazole are the drugs of choice for treating *Giardia* infections (Savioli *et al*., 2006). Interestingly, these compounds are ineffective on *Giardia* cysts (Harris *et al*., 2001); they affect *Giardia* trophozoites through the free radical produced when they are metabolized (Harris *et al*., 2001). Treatment failures, for unknown reasons, have been reported with all the commonly used drugs (Monis and Thompson, 2003; Müller *et al*., 2008; Pal *et al*., 2009; Savioli *et al*., 2006) and drug resistance to metronidazole in vivo (Johnson, 1993; Upcroft and Upcroft, 1993) and in vitro (Pal *et al*., 2009; Müller *et al*., 2007; Upcroft *et al*., 1996; Townson *et al*., 1994) have been reported.

### 1.1 *Giardia* Life Cycle

*Giardia* has two life cycle stages: non-proliferating infectious cysts, and proliferating trophozoites (Figure 1). Figure 2 shows the *Giardia* life cycle: infection begins when cysts are ingested; cyst exposure to the acidic pH of the host’s stomach induces excystation, producing a tetranucleate organism – the ‘excyzoite’– that divides within minutes into two vegetative binucleate trophozoites in the small intestine (Adam, 2001). Trophozoites proliferate and colonize the small
intestine sometimes causing malabsorption and diarrhea. Upon exposure to biliary fluid, some trophozoites differentiate into cysts, which are passed in the feces (Adam, 2001 and Svärd et al., 2003). Since the cyst wall allows the parasite to survive outside the host and initiate subsequent infection, Giardia’s encystment pathway serves as an important virulence factor.
Figure 1. Scanning electron micrograph (SEM) of *Giardia* trophozoites and a *Giardia* cyst.

(A) Trophozoites attached to the host’s microvillus brush border showing some of its eight flagella. (B) An intestinal villus colonized by *Giardia* trophozoites (C) Intact in vitro derived *Giardia* cysts (D) In vivo derived *Giardia* cyst undergoing excystation to release two trophozoites (Courtesy of Stanley Erlandsen).
Figure 2. Life cycle of *Giardia lamblia*.

The host is infected when viable cysts are ingested from fecally contaminated food, water, fomites, and host-to-host transmission. Cysts are not infectious immediately upon being excreted in feces; they may require a maturation period (Bingham et al., 1979). They can survive in cold conditions (<10°C) for several months and are resistant to normal disinfectants unlike the trophozoites, which are sensitive to changes in temperature, humidity, and ionic strength in addition to chemical disinfectants (Bingham et al., 1979). (Figure courtesy of Stanley Erlandsen)
1.2 Biology of *Giardia*

1.2.1 Cellular Structure of Trophozoites

*G. lamblia* trophozoites have a characteristic pear-shaped body measuring about 12 - 15 µm long by 5 - 9 µm wide. Trophozoites have a pair of symmetrical nuclei that are located in the anterior end of the parasite (Figure 3). *Giardia* nuclei have no nucleoli (Narcisi *et al.*, 1998), which suggests that transcription and processing of rRNA is not localized to specific regions of the nucleus. However, both of *Giardia's* nuclei are transcriptionally active, replicate at about the same time (Kabnick and Peattie, 1990; Wiesehahn, *et al.*, 1984), and contain approximately the same sequences and amount of DNA (Kabnick and Peattie, 1990).

Microtubular structures that make up the trophozoite's cytoskeleton include a median body, four pairs of flagella (anterior, postero-lateral, caudal, and ventral), and a ventral adhesive disk (VAD) (Figure 3). The flagella, originate from basal bodies that are located between the two nuclei (Tilney and Tilney, 1996), and are composed of nine pairs of microtubules that encircle two microtubules. The flagella are important for motility but not for attachment (Adam, 2001). The VAD, on the other hand, is the means by which trophozoites attach to the intestinal wall of their host in order to obtain required nutrients and avoid being transported out of the host by peristaltic movement of the intestine. The VAD is therefore a very important component of *Giardia's* cytoskeleton; it is made up of a set of microtubules that are linked to the ventral membrane, forming a base for the dorsal ribbons that extend
from the ventral membrane (Figure 3). Proteins found in the VAD include tubulins
(α and β) (Soltys and Gupta, 1994), the contractile proteins actin, myosin, and
tropomyosin (Feely et al., 1982) and Giardia's cytoskeletal proteins, giardins
(Peattie et al., 1989).

The median body is a cytoskeletal component that is specific to Giardia; it is
widely used for classifying Giardia species (Adam, 2001). As shown by light
microscopy, the G. lamblia median body is shaped like a claw hammer and each
trophozoite has one median body located in the midline between the ventral and
caudal flagella (Figure 3). The median body is made up of a group of microtubules
arranged in a tight bundle. Even though it is still not clear what the function of the
median body is, it has been proposed to be involved in VAD progenesis,
immobilization of microtubules between cell division and as a site for the assembly
of microtubules (Meng et al., 1996). The calcium binding protein caltractin/centrin
responsible for basal-body orientation, the VAD protein giardin and an
uncharacterized 101-kDa coiled-coil protein have been localized to the median
body (Belhadri, 1995; Marshall and Holberton, 1993).

Present in the trophozoite’s cytoplasm are lysosomal vacuoles, in addition to
glycogen and ribosomal granules; Golgi complexes have not been demonstrated in
vegetative trophozoites (Gillen et al., 1996) although Golgi-like structures appear in
encysting trophozoites (Luján et al., 1995a). Also present in the cytoplasm are
numerous mitochondrial remnants, mitosomes (Tovar et al., 2003) that are possibly
involved in the assembly of iron-sulfur clusters for ATP synthesis in the cytoplasm.
Figure 3. Scanning and transmission electron micrographs of *Giardia* trophozoites.

(A) Dorsal view of a trophozoite showing the relative positions of its four pairs of flagella; anterior, posterolateral, ventral and caudal. (B) The VAD and ventral flagella are visible in this ventral view of a trophozoite (Feely et al.; 1990). (C) A cross-sectional view of a trophozoite demonstrates the nuclei [N], flagella [F], vacuoles [V], and endoplasmic reticulum [ER] (Adam, 1991). (D) A cartoon of a *Giardia* trophozoite showing the median body, nucleus, flagella and ventral disk. (E) A close-up of the ventral disk shows the microtubules [MT] and dorsal ribbons [DR] (Adam, 1991).
1.2.2 Cyst Structure

*Giardia* cysts measure approximately 5-8 µm wide by 7-15 µm long with a cyst wall of approximately 0.3 to 0.5 µm thick. The cyst wall is made up of an outer filamentous layer and an inner membranous layer with the outer portion of the cyst wall covered by 7- to 20-nm diameter filaments that are arranged in a tightly packed meshwork (Erlandsen *et al.*, 1990; Erlandsen *et al.*, 1989; Feely *et al.*, 1984). Biochemical analyses show that these filaments of the outer cyst wall are made up of both carbohydrate and protein components (Jarroll and Paget, 1995; Manning *et al.*, 1992). The carbohydrate component, that is exclusively galactosamine in the form of a β-1,3-N-acetylgalactosamine (GalNAc) homopolymer called “giardan” (Sener *et al.*, 2009), accounts for approximately 63% of the weight of cyst wall filaments and the remaining 37% is made up of proteins (Gerwig *et al.*, 2002). The cyst wall filament proteins (Cwp) with molecular weights of 26 kDa, 39 kDa and 27 kDa have been identified as Cwp1, Cwp2 (Luján *et al.*, 1995b) and Cwp3 (Sun *et al.*, 2003).

Mature cysts have two sets of nuclei between which are located basal bodies and axial filaments (Adam, 2001). Two microtubular sheets are associated with the axial filaments and parallel rows of microtubules with perpendicular ribbon-like structures are randomly distributed in the cytoplasm. The ribbon-like structures and associated microtubules represent the disassembled VAD and flagella, which will reorganize upon excystation (Sheffield and Bjorvatn, 1977). No mitochondria,
endoplasmic reticula, or Golgi apparati are present in the cyst (Sheffield and Bjorvatn, 1977).

1.3. Differentiation

1.3.1 Cell Cycle

The cell cycle is a series of events during which eukaryotic cells divide into two daughter cells: it includes a G1 phase during which proteins required for DNA replication are synthesized, an S phase where DNA replication occurs, a G2 phase for accumulation of proteins required for mitosis and a M phase for splitting the cell and its genetic content into two distinct daughter cells. Giardia’s cell cycle plays an important role in its differentiation. During vegetative growth, Giardia trophozoites cycle between cellular ploidies of 4N and 8N (Figure 4) (Svärd et al., 2003; Bernander et al., 2001). At G1, trophozoites have a ploidy of 4N; each nucleus with a 2N ploidy. After DNA replication during S phase each nucleus acquires a 4N ploidy resulting in an 8N cell. This cell then undergoes nuclear division during the M phase to yield a cell with four nuclei, each with a ploidy of 2N. After cytokinesis, two cells are produced, each with two nuclei and a ploidy of 4N. The length of Giardia’s cell cycle ranges from 8-13 h depending on the strain (Reiner et al., 2008; Wiesehahn et al., 1984)
Figure 4. *Giardia* cell cycle.

Trophozoites cycle between a genome ploidy of 4N - at G phase of the cell cycle and 8N - after the S phase of the cell cycle. (Adapted from Bernander et al., 2001)
1.3.2 Excystation

In the mammalian host, excystation occurs with exposure to the contents of the upper small intestine after passage through the acidic environment of the stomach. Excystation was first achieved in vitro by exposure of cysts to an acidic pH 1.3 to 2.7 (Bingham and Meyer, 1979). Subsequent studies of excystation in vitro found an optimal pH of 4.0 (Boucher and Gillin 1990). In addition to an acidic pH, proteases are an important requirement for excystation; inhibitors of cysteine proteases prevented excystation without affecting trophozoite growth or replication (Boucher and Gillin, 1990; Ward et al., 1997). Calmodulin and protein kinase A (PKA) may also be involved in excystation (Bernal et al., 1998; Abel et al., 2001).

1.3.2.1 Excystation Events

Excystation is rapid; once cysts are exposed to conditions that promote excystation, the process is completed within 10 min (Buchel et al., 1987). Excystation entails detection of environmental stimuli across the cyst wall leading to highly coordinated molecular and structural responses (Svärd et al., 2003). After excystation is initiated, flagella appear through an opening in the cyst wall with subsequent release of an oval smooth cell body, the tetranucleate excyzoite with a ploidy of 4N in each nucleus (Buchel et al., 1987, Svärd et al., 2003). Rearrangement of the organism occurs, the excyzoite first becomes round, then elongated and flattened before undergoing one round of cytokinesis to produce two binucleate daughter cells with a ploidy of 4N in each nucleus (Figure 5). The two
daughter cells undergo another round of cytokinesis to yield four diploid trophozoites. Biochemically, a number of excystation-specific genes, many of which are variable surface proteins (VSP) are expressed (Hetsko et al., 1998; Svärd et al., 1998); these VSPs are involved in switching of Giardia's surface coat during differentiation (Svärd et al., 1998). Also, cysteine proteases, stored in lysosome-like peripheral vacuoles, are released into the space between the trophozoite and cyst wall to degrade the cyst wall during excystation (Ward et al., 1997).
Figure 5. Cell cycle events during encystment and excystation.

Trophozoites enter the encystment pathway from the M/G2 phase of the cell cycle (8N) and undergo one round of DNA replication to yield a 16N tetranucleate cell. Upon excystation, the 16N cell undergoes two rounds of cell division to yield a binucleate cell with a ploidy of 4N (Adapted from Bernander et al., 2001)
1.3.3 Encystment

Encysting trophozoites enter the encystment pathway from the G2/M phase of the cell cycle, after undergoing nuclear division but before cytokinesis, thus resulting in a cell with four nuclei. Even though there has been no consensus on the timetable for encystment due to variations in the data acquired by different researchers, encystment in *Giardia* can be divided into two phases - early and late (Adam, 2001). Upon induction of encystment, the first phase involves transduction of the encystment signal that results in synthesis and transport of cyst specific molecules. This is evidenced by the appearance of Golgi-like sub-organelles that label with NBD-ceramide, a biologically active fluorescent dye that is used to observe the morphology of the Golgi apparatus in living cells (Luján et al., 1995a). There is also an increase in ER proteins; Luján et al., (1996a) showed that the expression of the ER-resident chaperone BiP increased during encystment with an increase in other low molecular weight cyst proteins such as the Cwps. This suggests the involvement of the ER in encystment, possibly for the transport of cyst wall material. Encystment specific vesicles (ESVs), responsible for transport of cyst wall material to the plasma membrane, also appear in the early stages of encystment (Faubert et al., 1991; Luján and Touz, 2003). Morphologic studies indicate that these early events occur within 10 h after trophozoites are induced to encyst (Erlandsen et al., 1996). The late phase, which is completed by 16 h after induction (Erlandsen et al., 1996), involves changes in the cell structure (Figure 6) including rounding of the trophozoite, internalization of the flagella, external
assembly of the cyst wall, and one round of DNA replication to yield a cell with a ploidy of 16N (Figure 5). There is loss of motility during encystment and trophozoites are unable to attach to the surface of a culture flask in vitro and presumably the epithelium in the host’s gut. The ESVs disappear and the resulting cell contains two trophozoites with four nuclei that are yet to complete cytokinesis (Adam, 2001).
Figure 6. Field emission SEM of stimulation of *Giardia* encystment with high bile for 10 h (A).

Dorsal surface of a *Giardia* trophozoite obtained by indirect immunolabeling with of cyst wall antigen and 15 nm colloidal gold. The anterior [AL], posterior [PL], caudal [C], and ventral [V] flagella are shown. Initiation sites (shown with arrow heads) appear all over the surface of the trophozoite. Bar=1 µm. (B) Higher magnification of the dorsal membrane showing colloidal gold labeling (bright dots) for cyst wall antigen on projections from the cell’s surface. (C) Field emission SEM of stimulation of *Giardia* encystment with high bile for 14 h. Observe rounded cell studded with large cap like projections staining for cyst wall antigen. (Erlandsen et al., 1996).
1.3.3.1 Cyst Wall Synthesis

The only cyst wall sugar detected in *Giardia* is UDP-GalNAc. It is produced by an induced enzymatic pathway of enzymes and sugar phosphate intermediates (Macechko *et al.*, 1992; Sener *et al.*, 2004) (Figure 7). UDP-GalNAc is synthesized from fructose-6-phosphate, which is obtained from the glycolytic pathway. In the first step, fructose-6-phosphate is converted to glucosamine-6-phosphate (GlcN-6-P) by GlcN-6-P-deaminase (Gnp) (EC 5.3.1.10), an enzyme with both aminase and deaminase activity (Steimle *et al.*, 1997) but acting as an aminating isomerase in this reaction. This reaction is achieved in human and bacterial systems by the activity of L-glutamine D-fructose 6-phosphate amidotransferase (EC 2.6.1.16) (Kornfeld, 1967), which is lacking in *Giardia*. *Giardia* contains two forms of Gnp; Gnp-2 is constitutively expressed at low levels during the entire life cycle and Gnp1 is only expressed during encystment with a 13-fold increase in activity at 20 h after induction of encystment (Knodler *et al.*, 1999; Steimle *et al.*, 1997; van Keulen *et al.*, 1998).

GlcN-6-P acetyltransferase (Gna) (EC 2.3.1.4) completes the second step in the pathway by the acetylation of GlcN-6-P to produce *N*-acetylglucosamine-6-phosphate (GlcNAc 6-P). The activity of this enzyme increased 20-fold at 20 h after stimulation with bile (Macechko *et al.*, 1992). Phospho *N*-acetylglucosamine mutase (Agm) (EC 2.7.5.2) then converts GlcNAc 6-P into *N*-acetylglucosamine-1-phosphate (GlcNAc 1-P) to which a UDP moiety is attached by UDP-GlcNAc.
pyrophosphorylase (Uap) to produce UDP-$N$-acetylglucosamine (UDP-GlcNAc). The cyst wall carbohydrate UDP-GalNAc is then synthesized from UDP-GlcNAc by the action of UDPN-acetylglucosamine 4'-epimerase (Uae) (EC 5.1.3.7). All of these *Giardia* genes have been cloned, sequenced and expressed (Lopez et al. 2003). UDP-GalNAc is ostensibly converted to the homopolymer, giardan [1, 3 $\beta$ N-Acetyl Galactosamine]$_n$ by the action of cyst wall synthase (Karr and Jarroll, 2004; Sener et al., 2009).
Figure 7. Enzymatic pathway for the synthesis of N-acetylgalactosamine, the major carbohydrate portion of the cyst wall.

The enzymes are labeled as follows: 1) glucosamine-6-phosphate-isomerase; 2) glucosamine-6-phosphate N-acetylase; 3) phospho-N-acetylglucosamine mutase; 4) UDP-N-acetylgalactosamine pyrophosphorylase; 5) UDP-N-acetylgalactosamine 4' epimerase; 6) Cyst wall synthase
1.3.4 Regulation Of Differentiation

The physiological requirements for differentiation in *Giardia* have been at least partially characterized. Both encystment and excystation can be induced *in vitro* by modeling physiological stimuli; elevated pH and bile, and low pH and protease activities, respectively. However, there is little understanding of the molecular mechanisms governing regulation of *Giardia’s* gene expression during differentiation. Excystation and encystment are critical for the success of *Giardia* as a parasite. Understanding the mechanisms for their regulation will not only provide better insights into *Giardia’s* biology but will also provide better targets for developing drugs for the treatment and eradication of giardiasis.

There is little similarity between *Giardia* promoter regions and that of other known eukaryotic regulatory elements; regions needed for expression of *Giardia’s* genes are relatively short, <65 base pairs (bp) upstream from the start codon, and no classical TATA or CCAAT boxes or other cis-acting elements have been found in the promoters of many *Giardia* protein-coding genes (Elmendorf *et al*., 2001; Knodler *et al*., 1999; Sun and Tai, 1999; Yee *et al*., 2000). Only a few transcription factors have been identified and characterized to date in encysting *G. lamblia*. Sun *et al.* (2002) described a myeloblastosis (Myb) family transcription factor, Myb2, which is involved in coordinating up-regulation of its own expression and that of four other important encystment specific genes - Cwp1, 2, & 3 and Gnp during encystment (Sun *et al*., 2002). A homolog to the AT-rich interaction domain
(ARID) family of transcription factors has also been reported in *Giardia* (Wang *et al.*, 2007). ARID transcription factors are involved in regulation of cell proliferation and differentiation in higher eukaryotes; *Giardia* ARID1 (gARID1) served as an important trans-activator in regulation of the *Giardia cwp1* gene expression (Wang *et al.*, 2007).

Signal transduction clearly plays an important role in the regulation of *Giardia* differentiation: to survive and proliferate, *Giardia* trophozoites and cysts should be able to sense and react appropriately to their environments. Luján *et al.* (1996b) suggested that lipid signaling is an important feature of the signal transduction pathways induced during encystment, and Worgall *et al.* (2004) demonstrated that sterol regulatory element binding-proteins (SREBPs), gene transcription factors involved in lipid metabolism in mammalian cells, are involved in transcriptional regulation of encystment specific genes. The transcriptional activity of mSREBP is decreased by its ubiquitylation and/or sumoylation while phosphorylation by extracellular-signal regulated kinase (ERK)- increases transcription by mSREBP (Arito *et al.* 2008). *Giardia* homologues to the mitogen-activated protein kinases (MAPKs) -ERK1 and ERK2 - have been identified (Ellis *et al.*, 2003). Endogenous ERK1 exhibited increased activity, slightly higher phosphorylation and a noticeable partial relocalization to the cell membrane at an early time after induction of encystment while ERK2 relocates from the membrane to the cytosol with a decrease in its activity. These observations suggest the involvement of the MAPK-ERK signaling pathway in encystment.
1.4. Bistability

Bistability, a property of some regulatory pathways, has been described as important in the regulation of cellular processes such as differentiation and cell cycle progression in biological systems (Dubnau and Losick, 2006; Ferrell, 2002; Ferrell and Xiong, 2001; Smits et al., 2006). Put simply, bistability is the ability of a system to exist in alternate stable states. Regulatory pathways that exhibit bistability provide a mechanism to achieve heterogeneity in genetically homogenous systems of both prokaryotes and eukaryotes (Dubnau and Losick, 2006; Ferrell and Xiong, 2001; Smits et al., 2006). This type of heterogeneity allows populations to hedge their bets so that a proportion of the population enter a state that would be better adapted to a particular situation; survival of the species is therefore maximized (Dubnau and Losick, 2006; Kussell and Leibler, 2005; Thattai and Oudenaarden, 2004).

Bistable regulatory pathways also possess the ability to convert a graded input into a binary/all-or-none response due to the alternate states that arise, and the binary response generated can be either reversible or irreversible (Farrell, 2002; Kaern et al., 2003; Laurent and Kellershohn, 1999; Wang et al. 2006). This, as well as the switch-like behavior of such systems, ensures that regulatory pathways controlling important cellular processes are tightly regulated, especially for energy intensive and time consuming processes such as differentiation. Using modeling and
synthetic gene circuits, it has been shown that the co-occurrence of two or more phenotypically distinct subpopulations in a genetically homogenous culture is dependent on feedback-based wiring within the regulatory networks (Becskei et al., 2001; Gardner et al., 2000; Ozbudak et al., 2004; Smits et al., 2005; Veening et al., 2005). Two feedback mechanisms have been proposed to drive bistability: a positive feedback mechanism (Becskei et al., 2001) and a double negative feedback mechanism (Gardner et al., 2000) (Figure 8).

In a positive feedback loop (Figure 8a), a regulatory protein (R) promotes its own expression: when its expression is activated by a signal, the feedback loop is activated once R reaches a threshold level. As a result of the positive autoregulation, cells with more than a threshold amount of the regulator accumulate even more and exhibit the downstream effects of the regulator. A double negative feedback loop (Figure 8b) on the other hand involves a pair of mutually repressing regulators (R₁ and R₂): when the expression of R₁ is activated (by a physiological signal), it inhibits the expression of R₂ which in turn promotes more accumulation of R₁ because the inhibitory effect of R₂ on R₁ has been removed. Hence, until a new signal is presented to the pathway, it will remain in the R₁-on state. These two properties allow molecules in the pathway to respond in an ultrasensitive way.
Figure 8. Two proposed feedback mechanisms that promote bistability.

a. A positive feedback loop with transcriptional autoregulation; the regulator (R) promotes its own expression when the feedback loop is initiated (Adapted from Becskei et al., 2001). b. Double negative feedback; this requires two mutually repressing repressors. An inducer that promotes the expression of one repressor (R₁) that antagonizes the activity of another repressor (R₂) and keeps the system in an R₁-on state. Once the feedback loop is activated, the system will remain in a R₁–on state until a second inducer is introduced to initiate the expression of R₂ and convert the system into a R₂–on state, (adapted from Gardner et al., 2000).
Even though the regulatory elements that control encystment have not been elucidated, the properties of these pathways can be investigated. Bioinformatics and experimental studies suggest the involvement of regulatory pathways that can in principle promote bistability in *Giardia* due to the numerous positive and double negative feedback loops embedded in these proposed regulatory pathways (Ellis *et al.*, 2003; Sun *et al.*, 2002, Argüello-García *et al.*, 2009). In this study, I investigate the hypothesis that bistability exists in *Giardia lamblia*; the regulatory pathways induced during encystment have bistable properties. Specific objectives include:

1. Determine if *Giardia* maintains heterogeneity in its populations during encystment
2. Determine if members within *Giardia* populations contribute to the heterogeneity during encystment
3. Determine if the induction of encystment is reversible and if there is hysteresis in the expression of encystment proteins
4. Determine if cell-cell signaling molecules play a role in encystment.
CHAPTER 2: MATERIALS AND METHODS

2.1 General Procedures

(i) *Giardia* Culture and Encystment

*Giardia lamblia* strain WB clone 6 (American Type Culture Collection (ATCC), Manassas, VA) was grown axenically at 37°C in TYI-S-33 medium (Keister, 1983) without bile (growth medium) or supplemented with 1 mg ml\(^{-1}\) of bovine bile (Sigma) (low bile medium). Encystment medium was formulated by increasing the bile concentration in the growth medium to 10 mg ml\(^{-1}\) and adjusting the pH to 7.6-7.8 (Schupp *et al*., 1988). To induce encystment, the trophozoites were maintained in growth medium for at least 24 h and unless otherwise indicated, dividing trophozoites - which are motile and non-adherent - were removed by pouring off the supernatant of growing cultures. Non-dividing trophozoites, which adhere to the plastic culture flask in confluent cultures, were collected by chilling the culture flasks in an ice water bath for about 10 min. Cells were then transferred into encystment medium and incubated at 37 °C.

(ii) Protein Assay and SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed under reducing conditions using the Novex Tris-Glycine SDS-PAGE system from Invitrogen. Cells were harvested as described above and centrifuged at 2000 x g for 5 min. The pellet was washed 2X in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na\(_2\)HPO\(_4\), 1.47 mM KH\(_2\)PO\(_4\); pH 7.4) and incubated in 200 µl of lysis buffer (10 mM
magnesium sulfate, 50mm HEPES, 0.1%Titon X-100, pH 7.7). Protein concentration was determined by the Bradford method (1976) (BioRad) using a plate reader (BioTek Synergy HT). For SDS-PAGE, 20 µg of protein were diluted in 2x Novex Tris-Glycine SDS sample buffer (Invitrogen), 2x NuPAGE sample reducing agent (Invitrogen) and distilled water to a total volume of 25µl. The mixture was heated at 85 °C for 2 min to denature the proteins, allowed to cool and loaded on a 4-20% Tris-Glycine SDS-polyacrylamide gel (Invitrogen) in a Xcell SureLock™ Electrophoresis Cell (Invitrogen) containing 1X Tris-Glycine SDS running buffer (Invitrogen). Electrophoresis was performed for 1 h by applying 160 volts.

(iii) Western Blot Analysis

Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose membrane in transfer buffer (20 mM Tris, 150 mM glycine, and 20% (v/v) methanol) for 2 h at 20V in a semi dry transfer unit (Fisher scientific). The membrane was blocked with 5% (w/v) non-fat milk in Tris-buffered saline [25 mM Tris HCl (pH 7.4), 140 mM NaCl and 3 mM KCl] containing 0.1% (v/v) Tween 20 and then incubated overnight with the monoclonal antibody (mAb) for either Cwp 1 or 2 (generous gifts from Professor Hugo Luján)[1:1000 dilution in Tris-buffered saline]. The nitrocellulose membrane was washed for ca. 1 h in Tris-buffered saline containing 0.1% (v/v) Tween 20 before the secondary antibody (2º Ab), horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG (Fisher scientific) [1:20,000 dilution], was added. The membrane was incubated with the secondary
antibody for 1 h and washed 2X in Tris-buffered saline containing 0.1% (v/v) Tween 20. The membrane was then developed with ECL® (Invitrogen) and the chemiluminescent signals exposed to X-ray film (Kodak) which was developed with an Automatic X-Ray Film Processor (AFP) imaging system from ImageWorks (model #: Mini-Medical 90). A Novex sharp pre-stained protein standard from Invitrogen was used as molecular weight markers.

(iv) Immunofluorescence (IFA) and Flow Cytometric (FC) analyses

For either IFA or FC analyses, cells were harvested as described above, fixed in 3% formalin (diluted in PBS at pH 7.4) for 1 h and incubated in 3% formalin containing 0.02% triton X-100 for ca. 15 min. The cells were washed in 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄; pH 7.4) and incubated overnight with either the CWP 1 or 2 mAb. The mAb-labeled cells were washed twice in PBS and exposed to rabbit anti-mouse IgG- FITC conjugated 2° Ab for 1 h. The cells were washed twice in 1 X PBS and observed microscopically. For FC, a BD flow cytometer (Model #, FACScan) was used and data was analyzed with Flowjo v.7.2.4 (Tree Star Inc). Control samples were treated with only the FITC conjugated 2° Ab.

(v) Microscopy

Fluorescence microscopy was performed with a Zeiss Axioplan 2 compound microscope equipped with differential interference contrast microscopy (DIC). The microscope was equipped with a Hamamatsu C4742-95 camera used for taking all
pictures. For the single cell assays, an Olympus (IX71) inverted microscope with a Hamamatsu C4742-95 camera was used and an Olympus CK inverted microscope was used for cell counts performed with the hemocytometer.

(vi) RNA Extraction And cDNA Synthesis

RNA extraction was carried out using the Trizol Plus RNA purification kit from Life Technologies (USA). *Giardia* cultures were harvested by centrifugation and resuspended in Trizol reagent (1 ml Trizol 10⁻⁷ cells) for 5 min. Chloroform (200 μl for 1 ml of Trizol used) was then added to the cell lysate, vortexed for 30 s and incubated at room temperature for 2 min. The lysate was centrifuged at 13,000 x g for 15 min and the supernatant transferred to a clean tube. An amount of 70% ethanol equal to the volume of the supernatant was added and mixed vigorously for 1 min before incubation at room temperature for 5 min. An aliquot of the alcohol mixture was transferred into a spin cartridge, which was centrifuged at 13,000 x g for 15 sec. The filtrate was discarded and the process repeated until the alcohol mixture was depleted. The spin cartridge column was washed 2X with wash buffer 1 and 2X with wash buffer 2 by centrifugation at 13,000 x g for 15 seconds and dried by centrifugation at 13000 x g for 1 min. RNA was eluted with RNase free water and stored at 20 °C for future use. RNA quantification and purity were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific).

The purified mRNA (800ng) was synthesized into DNA using the QuantiTect reverse transcription kit from Qiagen (USA). Genomic DNA was eliminated
from the RNA sample with the gDNA Wipeout buffer at 42°C for 5 min. Reverse transcription was carried out at 42°C for 15-20 min and the reverse transcription mixture was then incubated at 95°C for 3 min to inactivate the reverse transcriptase.

(vii) Quantitative PCR (q-PCR)

Primer sets for detection of Cwp1 (cyst wall protein), Cwp2, and glutamate dehydrogenase (Appendix 1) were designed with Primer3 (http://frodo.wi.mit.edu/primer3) and were tested for specificity using a nBLAST homology search. The primers were synthesized by Fisher Scientific. Quantitative PCR (q-PCR) was carried out using RT² SYBR® Green/ROX FAST Mastermix (SA Biosciences) and an ABI 7000 detection system (Applied Biosystems). Each 25 µl PCR reaction contained 12.5 µl of the SYBR green mastermix, 60 ng of cDNA, 1.5 µl of 10 mM primer mix containing forward and reverse primers, and RNase free water. The PCR cycling conditions consisted 10 min of incubation at 95°C followed by 40 cycles of alternating temperatures of 95°C for 10 seconds and 60°C for 30 seconds. Fluorescence data were collected at the end of the 60°C annealing/extension step of each cycle. No-template and no-reverse-transcription controls were included in every assay and each sample was assayed in triplicate. A melting curve program was performed immediately after the PCR program.

2.2 Heterogeneity During Encystment
Trophozoites in confluent cultures were collected as described previously by centrifugation at 2000 x g for 10 min and induced to encyst for different time points (0, 3, 6, 12, 18, 24 and 72 h) in encystment medium. The percentage of encystment at each time point was determined by light microscopy and fluorescent microscopy using a polyclonal anti-cyst Ab. To determine if maintaining a heterogeneous population is an inherent property, the non-encysting cells in encysting populations were either subjected to another round of encystment or used to establish a confluent culture before a second round of encystment. The level of encystment was then determined.

2.3 Stability of *Giardia* Encystment

(i) Effect of replacing encystment medium with growth medium on a population of induced *Giardia* cells

Attached trophozoites (ca.10⁶) in exponential growth phase were induced to encyst with encystment medium for different time periods (0, 3, 6, 12, 18, 24 and 72 h) in 15 ml centrifuge tubes. After each time point, encysting cells were transferred into growth medium and kept in growth medium for a total of 72 h. The total number of cells and cysts were determined after each time point with a hemocytometer. Populations induced for just the time points indicated served as controls so that the populations in which encystment was interrupted (by replacing the encystment medium with growth medium) were compared to assess the effect of the interruption of encystment on cyst formation. To obtain the total number of cysts
formed, harvested cells were incubated in distilled water at 4°C for about 48 h, centrifuged at 2000 x g for 5 min to remove non-encysting and dead trophozoites, and resuspended in 1X PBS for cell count (Gillin et al., 1989). Fluorescent micrographs were obtained as described above.

(ii) Effect of replacing encystment medium with growth medium on individually induced Giardia cells

Using serial dilution, single cells from a population of confluent cultures were transferred into each well of a 96-well microtiter plate (BD Biosciences). Trophozoites were induced to encyst for different time points (3, 6, 12, 18, 24 and 48 h), centrifugation at 2000 x g for 5 min after which the encystment medium was removed by pipette. The encystment medium was replaced immediately with NB growth medium and incubated at 37 °C for a total of 72 h. The plate was analyzed by light microscopy after the induction period and after the 72 h time point to determine if cells had formed cysts (accessed by roundness of the cells, lack of motility and in some cases refractivity of cell membrane). Populations induced for just the time points indicated served as controls to which the populations where encystment was interrupted (by replacing the encystment medium with growth medium) were compared to access the effect of interruption.

2.4 Stability of encystment specific protein expression during encystment

The stability of cwp1 and cwp2 mRNA and protein expression was determined by
inducing trophozoites in their exponential phase of growth to encyst for different time points (0, 3, 6, 12, 18 and 24 h). After each time point, the cells were transferred from encystment medium into growth medium for a total of 24 h. Protein expression was determined in each sample by SDS-PAGE and Western blot analysis with CWP1 and CWP2 mAb (a gift from Professor Hugo Luján), and mRNA expression determined by q-PCR using α-tubulin and glutamate dehydrogenase as housekeeping genes and internal standards. Populations induced for just the times indicated served as controls to which the populations in which encystment was interrupted (by replacing the encystment medium with growth medium were compared, to assess the effect of interruption. FACScan analysis was also performed to determine the expression of these proteins in the individual cells.

2.5 Extracellular Signaling During Encystment

2.5.1 Co-culture of trophozoites with either encysting or non-encysting trophozoites

The effect of the two cell types obtained during encystment on fresh trophozoites was assessed to determine if encysting or non-encysting trophozoites play a role in the heterogeneity observed during induction of encystment. The objective was to determine if non-encysting trophozoites [NET] (in an induced population) or encysting trophozoites [ET] either promote or prevent encystment of induced trophozoites. Members of a 12 h, 18 h, 24 h and 48 h induced populations were co-cultured with trophozoites from a confluent non-induced population using a 12-well plate and corresponding 0.4 µm cell culture inserts (Corning, USA). To
obtain the NETs, which are usually found attached to the culture flask, the unattached cells (which contained ET and cysts in addition to dividing trophozoites) in the encysting culture were poured out and the flask was placed in an ice water bath for 10 min to detach the trophozoites. Detached trophozoites were washed 2x and resuspended in 1ml of growth medium and the cell concentration determined by hemocytometer count. The floating cell suspension was concentrated by centrifugation at 2000 x g for 5 min, washed 2x in growth medium and resuspended in 1ml of growth medium before the cell concentration was determined. The latter served as the committed cell/intact cyst population.

Co-culture was carried out at a 2:1 ratio of induced to fresh trophozoites; fresh trophozoites were kept in the wells of the culture plates while the ET and NET were kept in the cell culture inserts using the volume specification of the manufacturer. The ability of ET and NET cells to promote encystment was tested under non-inducing conditions in growth medium while their ability to inhibit cyst formation was tested under inducing conditions in encystment medium. The plates were covered and sealed with parafilm and incubated at 37 °C for 48 h. The cell suspension in the well plate was then analyzed for cyst formation. A control was included to verify that that there was no exchange of cells between the two populations separated by the insert: one had NET or ET in the insert but no trophozoites in the well and another had trophozoites in the well but no cells in the insert. The controls to which the co-cultured samples were compared were cyst formation without co-culture under inducing conditions and non-inducing
conditions: the inserts for these controls contained neither NET nor ET cells while the culture wells contained fresh trophozoites incubated in encystment medium and growth medium, respectively.

2.5.2 Induction of encystment with spent encystment medium

Encystment medium from populations induced to encyst for different time points (6, 12, 18, 24, 48 and 72 h) was collected by centrifugation and used in an attempt to induce encystment in trophozoites from a confluent growing population. The fresh trophozoites were induced for 12, 18, 24, and 48 h with the spent encystment medium, chilled on ice for 10 min and collected by centrifugation at 2000 x g for 5 min. The collected cells were then washed 2X in 1X PBS and analyzed for cyst formation as described above. Fresh trophozoites that were kept in encystment medium served as controls to which the spent medium induced encystment was compared for each time point studied. To counteract the effect of depleted lipids in the spent medium, a control experiment was included in which fetal bovine serum was added to the spent medium at a concentration of 10% before induction of encystment.
CHAPTER 3: RESULTS

3.1 *Giardia* populations exhibit phenotypic heterogeneity

Giardia populations were accessed for phenotypic heterogeneity during encystment induction and vegetative growth. Trophozoites were induced to encyst for different time periods and the level of encystment determined. When a population of trophozoites is induced to encyst, the total number of cells increases after the induction period, which indicates that some trophozoites enter the vegetative path of the life cycle even under encysting conditions. As shown in Figure 9(A), after 72 h in encysting conditions a significant portion (about 20%) of the population remained as trophozoites. When the non-encysting trophozoites (NET) in the induced populations were subjected to a second round of encystment, the population responded heterogeneously with some trophozoites encysting and others not (Figure 9C). The heterogeneity persisted when the NET were grown into confluent cultures before re-induction of encystment (Figure 9C). These observations indicate that trophozoites that did not encyst during the first cycle of encystment were still capable of encysting but remained undifferentiated, which kept the population heterogeneous. Figure 9A shows that heterogeneity is present in non-encysting populations as well. A small proportion of trophozoites in confluent vegetative populations encyst. To verify that this was not as result of depleting nutrients, the medium in a 72h vegetative population was changed every 24h; there was still a significant level of encystment observed (Figure 9A).
Analysis of individual encysting cells by flow cytometry also demonstrated that encystment specific protein expression is heterogeneous within encysting populations (Figure 10). Upon induction of encystment, the population bifurcates into high expressing and low expressing cells with regards to Cwp2 protein expression. This correlates with the observation that within encysting populations, some trophozoites enter the encystment pathway (encysting trophozoites [ET]) whiles others do not (NET). Figure 11 shows that remaining undifferentiated under encysting conditions is not beneficial to the individual trophozoites since non-encysting trophozoites become deformed and eventually undergo cell death after prolonged exposure to encysting conditions. This explains the reduced level of encystment obtain when NETs from a 72hr induced population were subjected to a second round of induction (Figure 9C).
Figure 9. Heterogeneity within Giardia populations.

(A). Effect of encystment medium on encystment of Giardia trophozoites: trophozoites induced to encyst exhibit phenotypic heterogeneity. Vegetative (non-encysting) Giardia populations are also phenotypically heterogeneous (0 h population). 72GM (growth medium) is a 72h non-encysting population whose medium was changed every 24h. (B). Differential interference contrast (DIC)
images of encysting *Giardia* population (400X oil): Black stars - trophozoites; black arrows - cysts (C). Encystment induction of non-encysting trophozoites in encysting populations: phenotypic heterogeneity persisted when the non-encysting trophozoites from encysting populations were subjected to a second round of encystment before and after being grown into a confluent culture, (2\textsuperscript{nd} Induction) and (R-ind) respectively. **: \( p < 0.05 \).
Figure 10. Encystment proteins are heterogeneously expressed during encystment.

(A). Flow cytometer analysis of CWP2 expression within *Giardia* populations induced for different time points. After 72 h of induction, a significant proportion of the population remained low-expressing cells (NET). (B). Light micrographs showing high (ET) and low CWP2 expressing cells (NET) (400X oil); upper panel: DIC micrographs of encysting trophozoites; lower panel: corresponding fluorescent micrographs showing high CWP2 containing trophozoites.
Figure 11. Non-encysting trophozoites after prolonged exposure to encysting conditions.

After five days in encysting medium, trophozoites become deformed. Column A shows normal trophozoites while B and C show deformed and lysed trophozoites respectively. Upper panel: DIC, lower panel: DAPI stained, *Giardia*’s characteristic nuclei (shown by white arrows) are absent from the cell undergoing cell death but present in the deformed and normal cells. Also shown are some of *Giardia*’s flagella (black arrows)
3.2 Trophozoites induced to form cysts become committed to encystment

Commitment to encystment was measured by determining the point at which transfer of encysting trophozoites into non-inducing conditions fails to prevent cyst formation. This in turn was elucidated based on increased cyst formation after interruption of encystment. The total number of cysts formed increased when encysting cells were transferred into non-inducing conditions (Figure 12) for all the time points studied. This observation led to the hypothesis that encystment involves a commitment point after which encysting trophozoites go on to form cysts even in the absence of encysting conditions. When a population of trophozoites is induced to encyst, some trophozoites do not enter the encystment pathway (Figure 9); these are able to slowly multiply resulting in a gradual increase in the total number of cells over time. To determine the effect of interrupting encystment on the ability of encysting trophozoites to resume growth, the total number of cells produced after replacing the encystment medium with growth medium was determined. There is an increase in the total cells formed after interruption of encystment for all the time points studied (Figure 13). This increase in total number of cells formed was most dramatic for the 3 h and 6 h time points, which showed about 23 and 19-fold increase in total cells produced, respectively (Figure 13B). Transfer of the 12 h encysting cells into growth medium resulted in only a 9-fold increase in total cells produced, after which the increase became gradual for the subsequent time points (Figure 13B).
The level of commitment was determined by expressing the total number of cysts formed at the end of the interruption time point as a percentage of the total cysts formed in the control population (trophozoites kept in encystment medium for 72 h). In all cases, there were trophozoites committed to encystment. Figure 14 shows that trophozoites induced to encyst become committed to encystment after a short period of time; by 12h after induction of encystment, virtually all of the cells were committed even though there were cells committed to encystment in all the time points studied. Although this data shows that trophozoites become committed to encystment, it does not show if trophozoites that were encysting at the time of interruption of induction were the same ones that eventually went on to form the cysts counted after the interruption time point.

To verify that trophozoites encysting at the time of interruption of encystment induction really go on to form cysts, a single cell assay was conducted in which encystment was interrupted in individual trophozoites induced to encyst for different time points. Trophozoites were then assessed for encystment at the end of the experiment. Cysts were identified based on the presence of encystment specific vesicles and refractivity of the cyst wall. Trophozoites identified as encysting based on the presence of vesicles usually went on to form cysts and just as in the bulk cell assay (Figure 13), the number of trophozoites committed to encystment increased with time (Figure 15). The single cell assay demonstrated that trophozoites become committed to encystment after a short period of time (Figure 15); by 6 h after induction of encystment, a substantial amount of trophozoites were committed to
encystment while the 3 h population showed only slight commitment.

**Figure 12. Effect of interruption of encystment on cyst formation.**

Interruption of encystment does not prevent cyst formation in induced trophozoites. Induction of encystment was interrupted and the total cyst formed determined as described in methods. The total cysts formed increased in all the ‘encystment interrupted’ populations (Induction interrupted) as compared to the amount of cyst formed after just the induction time points (Induced only).
Figure 13. Non-encysting cells resume growth after interruption of encystment.

(A). Effect of interruption of encystment on growth of encysting trophozoites: this shows the effect of interruption of encystment (with growth medium) on the ability of encysting trophozoites to resume growth. The total number of cells formed after interruption of encystment reduced over time, indicating that the number of cells capable of multiplication also reduced with longer exposure of trophozoites to encystment medium. (B). NETs multiply after interruption of encystment: the difference in total cells formed between the induced only and the induction
interrupted population is expressed as a percentage of the control (induced only) for each time point.
Figure 14. Commitment to cyst formation after interruption of encystment: effect of time of induction on number of cysts formed after interruption of induction.

The level of commitment to encystment was determined by expressing the total cysts formed after interruption as a percentage of the cysts produced in the population that was kept in encystment medium for the duration of the experiment (72h population - 72 h population – was assumed to be 100% commitment but actual encystment was 76%). Trophozoites were committed to encystment after a short period of time and most encysting trophozoites become committed to encystment after 12 h in encystment medium.
Figure 15. Commitment to encystment in individually induced trophozoites.

Encystment in individually induced trophozoites was interrupted after induction in encystment medium for different time points and the level of encystment was determined. Compared to encystment with interruption for each time point indicated (Induced only), encysting trophozoites become committed to encystment, mostly during the early stage of encystment. For the ‘Induction interrupted’ populations, the encystment medium was replaced with growth medium for a total of 72 h.
3.3 Encystment specific protein expression is stably maintained during encystment

In order for encysting trophozoites to become committed to the encystment pathway, the expression of the proteins required for the formation of cysts should be stably maintained once induced. To determine if expression of encystment specific proteins becomes committed during encystment, the expression of \textit{cwp1} and \textit{cwp2} in encysting trophozoites whose encystment has been interrupted was accessed by q-PCR and Western blot analysis. Figure 16 shows that transfer of encysting populations into non-inducing conditions does not cause an interruption in the expression of encystment specific proteins; after 6 h in encystment conditions, CWP1 and CWP2 protein expression increased in the absence of inducing conditions.
Figure 16. Effect of interruption of encystment on encystment specific protein expression.

Western blots showing CWP1, CWP2 and α-tubulin [as control] levels in extracts of encysting trophozoites taken at different times during encystment. When encysting trophozoites were transferred into non-inducing conditions to interrupt induction, the expression of encystment specific proteins, cyst wall protein 1 and 2 (CWP1 and CWP2) continued while that of α–tubulin expression remained unchanged. I-induction time, NI-time in growth medium after interruption of encystment.
The effect of interruption of induction on encystment specific protein expression was also accessed by flow cytometry where FITC conjugated secondary antibody was used to determine the presence of CWP2 in individual cells (Figure 17). After induction of encystment, CWP2 protein accumulation within cells becomes significantly evident by 12 h (indicated by the shift of the 12 h peak towards the right), even though there are is no significant amount of fully formed cysts by 12 h (Figure 17). The appearance of cysts in the 12 h ‘encystment interrupted’ population (12b₂) (Figure 17A) however indicates that these encysted trophozoites were committed to differentiate at the time of interruption and went on to form cysts after removal of the encystment medium. Subsequent time points also showed commitment; the percentage of high fluorescent cells produced after interruption of encystment increased in the 18 h and 24 h ‘encystment interrupted’ populations. In contrast to encystment specific proteins, expression of α–tubulin apparently was unaffected by interruption of induction; the protein levels stayed relatively constant between the induced only and encystment interrupted populations (Figure 16) and in individual cells the fluorescence distribution of the population remained unchanged for all the populations (Figure 17).
Figure 17. Commitment in the expression of encystment proteins in individual trophozoites.

Flow cytometry was used to access commitment in the expression of CWP2. Encysting populations whose encystment was interrupted after induction for
different time points were accessed for expression of CWP2 by indirect immunofluorescence using a monoclonal antibody and a FITC conjugated secondary antibody. (A). CWP2 expression (B). α–Tubulin expression.

For each time point I represents the induction time and NI represents time in growth medium after interruption of encystment. 0 was the starting population and 72I is a control population that was kept in encystment medium for 72h. (i = Histogram plots, ii = Scatter plots).
To determine if encystment protein expression is stably maintained at the level of transcription, q-PCR was used to assess mRNA levels before and after interruption of induction. Unlike the protein expression data from the Western blot assay, the data presented here show that \textit{cwp} gene expression occurs during the early stages of encystment (Figure 18A). However, upon interruption of encystment, gene expression is stably maintained only during the late phase of encystment. As shown in Figure 18B, relative to the expression of glutamate dehydrogenase, encystment specific gene expression is stably maintained after 18 h in encysting conditions; interruption of encystment after 3 h and 6 h results in interruption of \textit{cwp1} and \textit{cwp2} gene expression.
Figure 18. q-PCR analysis of cwp expression during and after interruption of encystment.

(A) Electrophoresis of PCR products after q-PCR analysis for cwp expression in induced (b₁) and induction interrupted (b₂) populations. DNA ladder is shown in column 1; i = cwp₁; and ii = cwp². 24B was kept in encystment medium for 24hrs.

(B). Using glutamate dehydrogenase respectively as a reference gene, relative
expression of cwp was quantified. Data were analyzed by the Delta-Delta Ct method for relative quantification as described previously (Pfaffl, 2001).
3.4.1 Intercellular communication during encystment

Extracellular signaling is an important means for regulating differentiation in both prokaryotes and eukaryotes. The involvement of extracellular signaling molecules in *Giardia* encystment induction was accessed with spent medium from *Giardia* populations that had been induced to encyst for 6, 12, 18, 24, and 72h. The encystment levels obtained from the use of spent encystment medium were not statistically different from those obtained when fresh encystment medium was used for inducing encystment. As shown in Figure 19A, induction of encystment with spent encystment medium yielded relatively similar levels of encystment as induction with fresh encystment medium ($p<0.05$). To verify that the encystment obtained using the spent medium was not necessarily due to cholesterol depletion, cholesterol was replenished in the spent medium by adding 10% calf serum. The encystment level obtained when serum was added to the spent encystment medium was comparable to that obtained with just spent encystment medium (Figure 19B) and statistical analysis indicates that there was no significant difference between the two outcomes.
Figure 19. Induction of encystment with spent encystment medium.

(A) Spent encystment medium from populations induced for different time points (6, 12, 18, 24, 48 and 72 h) was used to induce encystment of fresh trophozoites for 18, 24, 48 and 72 h. (B) This shows the effect of adding fetal calf serum to spent encystment medium on the level of cyst observed. Serum was added to replenish lipids and cholesterol.
A co-culture technique was employed to determine if either encysting or non-encysting trophozoites, in induced populations, influence encystment by secreting extracellular signaling factors. Figure 20 (A) and (B) show the effect of encysting trophozoites (ET) on encystment under non-inducing conditions (co-culture) and the effect of non-encysting trophozoites (NET) on encystment levels under both inducing and non-inducing conditions. The encystment levels obtained when cysts were co-cultured with trophozoites were not statistically different from those obtained without co-culture. Similarly, compared to induction of encystment without NET, co-culturing NET with induced trophozoites does not result in significant changes in the levels of encystment obtained. These observations suggest that the sub-populations that arise during induction of encystment have no significant involvement in the levels of encystment observed; cysts, trophozoites committed to encystment and non-encysting trophozoites within encysting populations appear to be incapable of inducing encystment on their own. Induction of encystment in _G. lamblia_ apparently does not involve intercellular communication.
Figure 20. Effect of ET and NET on encystment.

Co-culture of members encysting populations with fresh trophozoites for 24 h (i) and 48 h (ii). (A) Encysting trophozoites and cysts (ET) from a 24h induced population were co-cultured with trophozoites under non-inducing conditions (B) Co-culture of non-encysting trophozoites from a 24h induced population with fresh trophozoites under both inducing and non-inducing conditions. (p>0.05 in all cases)
CHAPTER 4. DISCUSSION

Cell differentiation is a fundamental event in the development of unicellular and multicellular organisms. In most unicellular organisms, the transition from growth to differentiation is an important process that mediates responses to harsh conditions such as nutrient starvation and unfavorable growth conditions. As a lipid auxotroph (Jarroll et al. 1981), Giardia responds to cholesterol starvation, such as can occur in the lower small intestine, by differentiating into cysts (Luján et al. 1996a), which allow for its survival outside the host and for its transmission from one host to the other. The environment colonized by Giardia is one with constantly changing conditions and Giardia must respond rapidly and appropriately to these changes to be successful as a parasite. For example, if trophozoites entering the duodenum during an infection encyst before they reach the jejunum, they forfeit the opportunity to divide and increase the probability of propagating the infection. On the other hand, trophozoites that are carried downstream must rapidly differentiate into cysts to be able to survive outside its host and reach another host. Despite the fact that we know a great deal more about Giardia encystment than that of any other protozoa, there are still many important questions to be answered. For example, it is still not clear how physiological signals are transduced from the cell surface into the cell for the activation of the cell’s differentiation machinery and the mechanisms governing regulation of the decision-making processes that make Giardia a successful parasite are still unclear.
Described in this dissertation are events that indicate that some components of the encystment regulatory pathways have bistable properties that ensure the decision to differentiate is tightly controlled in order to maximize parasitism. This dissertation aimed to answer the following questions: 1) Does induction of encystment result in an all or none outcome? 2) Is induction of encystment reversible? 3) Finally, extracellular signaling during encystment was investigated to understand the nature of the signaling mechanisms employed during encystment.

4.1 Heterogeneity during encystment

It is becoming increasingly apparent that cells in a clonal population do not respond uniformly when subjected to a particular treatment (Smits et al. 2005; Veening et al. 2005; Balaban et al. 2004, Henderson et al. 1999; Kim and Weiser, 1998). This kind of heterogeneity, like that resulting from genetic rearrangement in phase variation, is an additional mechanism adopted by many unicellular organisms for increased survival in their ever changing and unpredictable environments (Dubnau and Losick, 2006; Booth, 2002; Sumner and Avery, 2002; Moxon et al. 1994; Zieg et al. 1977). For example, Pilawal et al. (2007) showed that bimodality in gene expression during pheromone response allows a yeast cell population to diversify its transcriptional response at relatively low pheromone concentrations thus reducing the cost of possible inappropriate engagement in expensive pheromone-dependent gene amplification. Also, when conditions that trigger sporulation or competence are applied to a Bacillus subtilis culture, only part of the population
undergo either process leading to two clearly distinguishable cell types, a phenomenon that maximizes the fitness of the species (Cahn and Fox, 1968; Hadden and Nester, 1968; Chung et al. 1994).

*Giardia* trophozoites respond heterogeneously when induced to encyst, some members of the population enter the encystment pathway while others remain in the vegetative pathway. Since cyst formation is a survival strategy, when *Giardia* trophozoites are faced with adverse conditions, lack of encystment would be detrimental. Thus it was interesting to observe that even after 72 h in encysting conditions, a significant proportion of the population remained as trophozoites (Figure 9). *Giardia* trophozoites appear to enter the encystment pathway from the G2 phase of the cell cycle and once encystment is initiated it is completed by 18 hours (Bernander et al. 2001, Erlandsen et al. 1996). Reiner et al. (2008) determined the length of the cell cycle for *Giardia* strain WB C6 to be 8 hours which means that exposing trophozoites to encystment conditions for 72 h allows enough time for cells that were not in the G2/M phase of the cell cycle at the time of induction to complete the cell cycle and enter the encystment pathway. Increased cell concentration after induction of encystment however suggests that some trophozoite forfeit the opportunity to encyst in favor of vegetative growth. One could argue that this is true simply because these trophozoites cannot encyst. However, when the trophozoites that did not encyst were collected and exposed to another round of induction, they again gave rise to a heterogeneous population, as did inducing the progeny of the non-encysting trophozoites. This shows that the
non-encysting trophozoites from the first induction had the ability to encyst but for some as yet unknown reason, did not. Prolonged exposure of these non-encysting cells to encystment medium causes non-encysting trophozoites to become deformed and eventually undergo cell lysis. It is, therefore, clear that the decision to remain a trophozoite in encysting conditions is inherent in the population and is likely a mechanism for giving the parasite population a chance to re-colonize that particular host should the stressing event pass quickly enough.

Gene content analyses have shown that a trophozoite population in the log phase of growth consists of about 70% of cells in the G2/M phase of the cell cycle (Bernander et al. 2001; Reiner et al. 2008) suggesting that when such a population is induced to encyst, one should expect about 70% of encystment by 24 h of induction. Such an encystment rate has never been reported indicating that heterogeneity in encysting conditions may be an inherent property of Giardia. Induction of encystment under lipoprotein-free conditions resulted in about 50% encystment after 24 h, which also shows that even in conditions with absolute lipid deprivation, encysting populations remained heterogeneous (Luján et al., 1996b).

Heterogeneity was observed in vegetative cultures as well; there were significant numbers of cyst in non-encysting populations, indicating a stochastic basis for encystment. The presence of the two cell types in both encysting and non-encysting conditions exemplifies bistable populations (Fujita et al. 2005; Maamar and Dubnau 2005; Smits et al. 2005; Veening et al. 2005); and the persistence of
heterogeneity after several cycles of induction or vegetative cultivation indicates that maintaining a bistable population is an inherent property of *Giardia*.

The decision by some members of a *Giardia* population to either take the vegetative phase of the life cycle under encysting conditions or encyst under vegetative conditions is a strategic one employed by *Giardia* to increase the fitness of its populations. Cyst formation and excystation are both metabolically expensive processes that require synthesis and degradation of proteins and carbohydrates. When faced with encysting conditions, cells that do not encyst are in a position to resume rapid growth, without the expense of either encystment and/or excystation, if conditions become favorable. In vivo, trophozoites that emerge from the cyst initiate encystment when they enter the small intestine. Trophozoites that do not initiate encystment either face the danger of being washed out of the host down the intestinal tract or have the ability to colonize the small intestine and divide to produce more trophozoites that can later encyst, thus increasing the chances for survival of the parasite.

**4.2 Commitment to encystment**

The bistability observed within biological systems is usually associated with hysteresis; that is, the ability of the system to maintain, in a sustained manner, a particular state despite the fact that the stimulus initiating this state is no longer present or is below the level that initially activated the system (Dworkin and
Losick, 2005; Ferrell and Xiong, 2001; Wang et al. 2006). Commitment to differentiation in unicellular organisms has been extensively studied in both prokaryotic and eukaryotic systems where the phenomenon has been attributed to hysteresis in the regulatory pathways employed (Wang et al. 2006; Sha et al. 2002; Bagowski and Ferrell, 2001). Considering that Giardia trophozoites, like most unicellular organisms, must respond to a constantly changing environment, it is important that they respond rapidly to the appropriate signals in order to survive in their environments. Furthermore, the synthesis of N-acetylgalactosamine is costly and energy expensive: it takes place at the expense of continued growth, substrate level phosphorylation of ADP, and requires the use of UTP for cyst wall synthesis. Additionally encystment requires an elaborate sequence of gene expression to synthesize encystment related proteins. The process must therefore be tightly regulated to ensure that it only takes place when absolutely necessary. For example, if after a trophozoite has entered the encystment pathway, conditions become favorable, the regulatory mechanism employed should allow the trophozoite to resume growth, and in situations where the unfavorable conditions become prolonged, encystment needs to be completed in order for the trophozoite to survive. The use of signaling pathways that can switch back and forth between alternate states and also remember a signal even after it has been removed will allow for tight regulation of these processes (Bagowski and Ferrell, 2001; Xiong and Ferrell 2003; Smits et al. 2006).

As shown by flow cytometry, the expression of CWP2 within Giardia populations
in response to high bile conditions is an all or none phenomenon; some cells express encystment specific proteins while others do not under conditions for encystment. Such a response can either arise from an ultrasensitive, monostable signaling system or from a bistable signaling system (Bagowski and Ferrell, 2001). My data show that the encystment pathway can be turned on spontaneously which suggests the involvement of a switch-like regulatory system. To investigate if the bistability observed results from hysteresis of the regulatory pathways induced during encystment, I studied commitment of Giardia trophozoites to encystment.

The data presented here show that there is a point of commitment during encystment after which encysting trophozoites complete the encystment process even in the absence of the encystment medium. Even though commitment was observed at all time points, the majority of the population became committed to the encystment pathway after 12 hours in encysting conditions. The total number of cells produced after interruption of encystment indicates that growth was resumed after the cells were presented with non-inducing conditions which also shows that some members of the population were not committed to the encystment pathway by the time the encystment medium was removed and could therefore resume rapid growth when conditions became favorable. The ability of induced populations to resume vegetative growth was drastically reduced after 12 h in encysting conditions and correlates with the observation that the majority of encysting trophozoites pass a ‘point of no return’ in the encystment pathway after 12 h. Interestingly, the 12 h ‘point of no return’ correlates directly with the decrease observed in the ability of
exogenous glucose to stimulate oxygen uptake and with the appearance of mRNA for key enzymes required for the synthesis of cyst wall carbohydrate, (Lopez, et al. 2003; Paget, et al. 1998).

Encystment specific protein synthesis is also stably maintained during encystment; there is increased synthesis of CWPI and CWP2 after interruption of encystment. In most cases, the proteins were detected at 12 h after induction of encystment, and the expression levels increased to levels similar to the control, which was left in encystment medium for 24 h. Even though cyst wall proteins were detected in the late phase of encystment by Western blot (Luján et al. 1995b), mRNA for these proteins appears quite early (Luján et al. 1995b; Morrison et al. 2007); transcription of cyst wall proteins occurs by 3 h after induction of encystment. However, in contrast to protein synthesis, mRNA production reduces after interruption of encystment which suggests that the mRNA present at the time of interruption of encystment was transcribed into protein and eventually used to form functional cysts; cyst production increased after interruption of encystment. These observations show that commitment to encystment with respect cwp expression occurs during the late phase of the induction process (Erlandsen et al., 1996), and this explains the observation that most members in the population were committed to encystment after 12 h in encystment medium. It also suggests that during the very early stages of encystment, trophozoites may be capable of aborting the encystment process when the stressor is removed and conditions once again become favorable for growth. This indicates the involvement of regulatory
pathways with switch-like properties that can be spontaneously activated to promote encystment and once activated can be repressed should conditions become favorable.

Considering that the mRNA for the enzymes in the cyst wall carbohydrate pathway become apparent late after induction of encystment (van Keulen et al., 1998), it appears that the expression of cyst wall proteins and their synthesis precedes that of the cyst wall polysaccharide into which they become incorporated to form a functional cyst wall. The data also suggest that cyst wall proteins assembly and transport are requirements for commitment to encystment since commitment to encystment occurred after the appearance of ESVs. As reported earlier (Davids et al. 2004; Reiner et al. 2001; Tuoz et al. 2002), substances that inhibit formation of ESVs prevent differentiation of trophozoites into cysts, indicating that transport and assembly of cyst wall proteins is a key regulatory step during the encystment pathway.

4.3 Extracellular cell signaling during encystment

Cell–cell signaling mediated by diffusible molecules play an important role in regulating many physiological processes in prokaryotic and eukaryotic organisms. In multicellular organisms, extracellular signaling molecules function as hormones and neuromediators and in eukaryotic microorganisms such as slime molds intercellular signaling molecules coordinate the aggregation of
cells for sexual mating and differentiation. Quorum sensing represents a common cell-cell signaling phenomenon in bacterial systems. As in some eukaryotic microorganisms, quorum sensing allows bacteria to function as multicellular organisms; enabling bacterial populations to reap benefits that cannot be achieved by individual members acting alone.

Maintaining heterogeneity within Giardia populations increases the chances of Giardia’s survival and since maintaining heterogeneity appears to be an inherent property of Giardia populations, it was of interest to determine if individual members of encysting populations play a role in the heterogeneity observed during encystment. A co-culture approach to determining the effect of cysts and non-encysting trophozoites (in induced populations) on trophozoites under both inducing and non-inducing conditions showed that when trophozoites are co-cultured with cysts or trophozoites committed to encystment, encysting conditions are still required to achieve a statistically significant level of encystment and under inducing conditions, the cysts/committed trophozoites did not inhibit cyst formation in the trophozoite population with which they were co-cultured. Also shown by these studies was evidence that the non-encysting trophozoites within encysting populations have no apparent influence on the level of encystment observed in the trophozoite population with which they were co-cultured. These observations suggest that there is no obvious intercellular communication or signaling involved in the induction of encystment. To shed more light on this, the effect of spent encystment medium on cyst formation was also investigated. The
level of encystment achieved with the use of spent encystment medium from induced populations is not significantly different from that obtained with the use of regular encystment medium which supports the idea that there are no extracellular signaling molecules produced during encystment. Liquid chromatographic analysis has shown that spent encystment medium lacks cholesterol (Jarroll et al., 2011); induction of encystment with such a medium should in principle result in close to a 100% encystment levels due to the importance of cholesterol to trophozoites (Luján et al. 1995a) and replenishing cholesterol in spent encystment medium should essentially prevent cyst formation. The data presented show that under conditions that are considered unfavorable to Giardia trophozoites the encysting populations remain heterogeneous; a 100% encystment was not achieved with any of the spent encystment medium used and induction of encystment in spent medium with added cholesterol/liporoteins in the form of calf serum did not prevent cyst formation. This could be due to the persistent effect of the high bile and high pH conditions initially present; pH analysis showed that the pH of the spent medium remained unchanged.
CHAPTER 5. CONCLUSION

With the use of techniques that allow for the study of individual members of *Giardia* populations, I have shown that *Giardia* populations bifurcate into subpopulations of the two *Giardia* cell types (cysts and trophozoites) under both vegetative growth and encysting conditions. The heterogeneity observed is employed by *Giardia* most likely to maximize its chances of survival; the presence of both cell forms in a population allows the population to survive under both favorable and unfavorable conditions and at a lower energy expense. I investigated the role of the sub-populations produced during induction of encystment in promoting the heterogeneity observed and the data suggest that in the presence of both sub-populations, encysting conditions are required to achieve statistically significant levels of encystment.

Even though it has been established that cyst formation is induced by nutrient deprivation (Jarroll *et al.*, 1981; Luján *et al.*, 1996b), the presence of cysts in vegetative growth conditions clearly shows that the regulatory mechanisms governing encystment can be spontaneously turned on, which suggests the involvement of random switching mechanism for the regulation of encystment. Random switching regulatory pathways that promote heterogeneity are controlled by a threshold mechanism; regulatory proteins in such systems must reach a critical concentration to initiate altered gene expression (Dubnau and Losick, 2006; Ferrell, 2002). As a result, cells in the population with this critical concentration exhibit the
phenotypic manifestation of the altered gene expression while the others do not. I propose that as in most bacterial systems that exhibit bistability, regulatory pathways for *Giardia* differentiation employ a threshold mechanism that ensures that cyst formation is initiated only in cells with the right concentration of the regulatory proteins, and due to differences in the rate of gene expression, some cells are able to reach the concentrations required while others are not. With such a mechanism in place, if conditions become favorable after a transient period of nutrient deprivation, members in the population with sub-optimal concentrations of encystment regulatory proteins will be able to resume growth without the energy expense of both encystment and excystation. The use of a threshold mechanism in the regulation of encystment will also ensure that when unfavorable conditions become prolonged, some trophozoites will be able to form cyst in order for the population to survive.

In addition to a threshold mechanism, feedback loops in regulatory pathways promote bistability (Ferrell, 2002; Ferrell and Xiong, 2001; Smits et al, 2005; Veening *et al*, 2005); when the concentration of a regulatory protein in a feedback loop reaches the critical concentration for activating the feedback loop, the feedback loop is turned on and its effect sustained. Once this is achieved, the downstream effect of the regulatory pathway is expressed even in the absence of the inducing signal- a mechanism described as hysteresis. The data presented here suggest that there is a point during induction of encystment when hysteresis is established; trophozoites become committed to encystment after a short period of
time although the majority of trophozoites become committed during the late phase of encystment. There is evidence for the involvement of feedback loops in regulation of encystment; *Giardia* transcription factor, Myb2, promotes up-regulation of its own expression and that of four other important encystment specific genes - cwp1, 2, & 3 and Gnp - during encystment (Sun *et al*., 2002). The MAPK-ERK signaling pathway proposed for regulation of encystment (Ellis *et al.*, 2003) also has numerous feedback loops embedded in it (Ferrell, 2002; Xiong and Ferrell, 2003). The involvement of feedback regulatory loops that exhibit hysteresis will explain the stability in the expression of encystment specific proteins; both the transcription and translation of cyst wall proteins is sustained after removal of the inducing signal by 12 h into encystment. This ensures that upon induction of encystment, only cells that accumulate the necessary concentration of regulators for activating the encystment regulatory pathways will go on to encyst. Hence in addition to promoting heterogeneity, feedback loops in the regulatory pathways induced during encystment ensure that when encysting conditions are experienced only transiently, the encystment process can be aborted in favor of vegetative growth.

In addition to cyst formation in *Giardia lamblia*, bistability, mediated by random switching mechanisms, threshold mechanisms, as well as hysteresis, play an important role in *Giardia’s* survival.
REFERENCES


Fujita, M., and Losick, R., (2005). Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. *Genes Dev.*, **19**: 2236–2244.


*Giardia*: a review. *Folia Parasitol.*, 42:81–89.

Metabolism In: Giardia, a model organism (Luján, H.D. and Svärd, S. Eds).
SpringerWienNewYork, pp, 127-137

9:183-186.

Kabnick, K. S., and Peattie, D. A., (1990). In situ analyses reveal that the two


amethylgalactosaminyltransferase activity is induced to form the novel GalNAc


Paliwal, S., Iglesias, P. A., Campbell, K., Hilioti, Z., Groisman, A., and


Ward, W., Alvarado, L., Rawlings, N. D., Engel, J. C., Franklin, C., and


APPENDIX 1: List of primers for qPCR

P2Cwp1(L): GAGAACATCGGCTGCCTTAC
P2Cwp1(R): CAGACCGGCACTATTGACCT
P2Cwp2(L): GGCATCACATGCGACTCTAA
P2Cwp2(R): ATTGAGGTGAGGGCACAAAG
P2GDH(L): GCGCTGTCTACTTCTGGAG
P2GDH(R): CAGCTTCTCCTCGTTGAAC

GDH = Glutamate dehydrogenase