Georgia State University

ScholarWorks @ Georgia State University

Biology Dissertations

Department of Biology

Summer 8-18-2010

Encystment of Acanthamoeba and Evaluating the Biobus Program

Brandi C. Trevisan

Follow this and additional works at: https://scholarworks.gsu.edu/biology_diss

 Part of the Biology Commons

Recommended Citation

Trevisan, Brandi C., "Encystment of Acanthamoeba and Evaluating the Biobus Program." Dissertation, Georgia State University, 2010.
doi: https://doi.org/10.57709/1406928

This Dissertation is brought to you for free and open access by the Department of Biology at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Biology Dissertations by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact scholarworks@gsu.edu.

ENCYSTMENT OF ACANTHAMOEBA AND EVALUATING THE BIO-BUS PROGRAM

by

BRANDI C. TREVISAN

Under the Direction of Sidney Crow

ABSTRACT

Acanthamoeba are ubiquitous protists that play an environmental role in regulating microbial diversity; they also occasionally cause infections of the eye (*Acanthamoeba* keratitis) and brain (granulomatous amoebic encephalitis). These organisms exhibit two distinct phenotypes. The trophozoite form dominates in favorable conditions, in which the *Acanthamoeba* move through the extension of pseudopodia, engulfing microbes and other particles. During stressful conditions, the *Acanthamoeba* undergo a process of encystment, in which they build a double cell wall and become relatively inactive. The cyst form can survive years until more favorable conditions arise, at which point they may excyst. For this study, multiple laboratory encystment methods were compared to

determine the percent encystment and the different viabilities of laboratory-produced cysts. Furthermore, four different encystment genes were targeted for development of a primer library for reverse-transcription, polymerase chain reaction expression studies. The library was developed using sequences accessed from various databases, including NCBI and EMBL; primers were screened through polymerase chain reaction, and those primers producing positive results were used to further screen cellular RNA that was extracted from encysting cells over various time points during the encystment process, and using various encystment media. Using these methods, target gene involvement in the encystment process was compared between species and encystment methods. These studies lay the foundation for quantitative gene expression analysis, and provide the basis for comparison of various encystment methods.

INDEX WORDS: *Acanthamoeba*, *Acanthamoeba* keratitis, Contact lens solutions, Cysts, Encystment, RT-PCR, Trophozoites

ENCYSTMENT OF ACANTHAMOEBA AND EVALUATING THE BIO-BUS PROGRAM

by

BRANDI C. TREVISAN

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2010

Copyright by
Brandi C. Trevisan
2010

ENCYSTMENT OF ACANTHAMOEBA AND EVALUATING THE BIO-BUS PROGRAM

by

BRANDI C. TREVISAN

Committee Chair: Sidney Crow

Committee: George Pierce

Barbara Baumstark

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

August 2010

ACKNOWLEDGEMENTS

I hereby bequeath my undying gratitude to ALL of my family members; your assistance and love was instrumental in my being able to perform this research and write this paper. Without all of you, big and small, I could not have done this.

Furthermore, I am forever indebted to my advisor, Dr. Sidney Crow, whose patience, guidance and inspiration (in his own way, of course) are without rival in the academic world. And to the Crow's nest (special folks, all of you) I leave you my bench, and my best wishes for the awesome careers you are all making for yourselves.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGE	agarose gel electrophoresis
AK	*Acanthamoeba* keratitis
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cAMP	cyclic adenosine monophosphate
CPE	cytopathic effect
CRCT	Criterion-Referenced Competency Test
DEPC	diethylpyrocarbonate
diH_2O	de-ionized water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
EOCT	End-of-Course Test
EMBL	European Molecular Biology Laboratory
EMSP	encystment mediating serine proteinase
EST	expression sequence tag
GAE	granulomatous amebic encephalitis
kDa	kilodaltons
MAPK	mitogen-activated protein kinase
MBP	mannose-binding glycoprotein
MIP	mannose induced protein
MPN	most probable number
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NNA	non-nutrient agar
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PYG	proteose peptone-yeast extract-glucose medium
RAPD	randomly amplified polymorphic DNA
rcf	relative centrifugal force
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription – polymerase chain reaction
SDS	sodium dodecyl sulfate
siRNA	small interfering ribonucleic acid
SLSP	subtilisn-like serine proteinase
SSU	small subunit
TSB	tripticase soy broth
V	volt

1 ENCYSTMENT OF ACANTHAMOEBA

1.1 INTRODUCTION

Acanthamoeba are free-living protists that have a two-stage life cycle. They can exist in trophozoite or cyst form, favoring the trophozoite form under non-stress conditions; in this state they are able to move through extension of pseudopodia, phagocytose particles and other organisms, and carry out cell division. The cyst structure that is formed under stressful conditions allows the organism to survive until more beneficial conditions arise, and is characterized by a shrinking and rounding of the cell, and the building of two distinct cell wall layers (Figure 1).

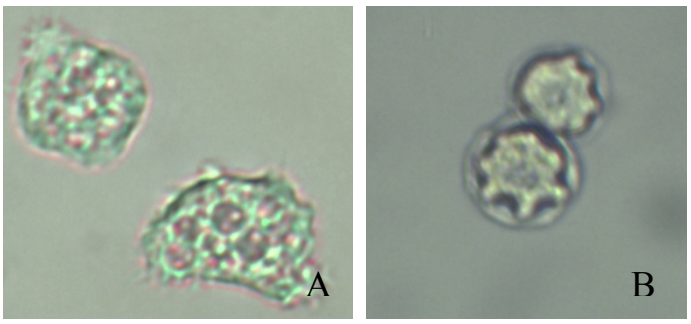

Figure 1. The trophozoite (A) and cyst (B) form of *Acanthamoeba castellanii*. Both images were taken at 400X magnification.

Acanthamoeba are ubiquitous organisms, found in soil, air, and water (De Jonckheere 1991). It is estimated that we inhale two of these organisms per day, and they have even been isolated from human fecal matter (Zaman et al. 1999).

As consumers of bacterial species in the soil, they regulate microbial diversity. As they do so, they excrete mineral waste; this becomes available for further growth of primary decomposers that might otherwise be limited by mineral availability (Khan

2009). Thus, these free-living amoeba play an instrumental role in the nutrient cycle, and even have a demonstrable effect on plant growth (Clarholm 2002).

Classification of *Acanthamoeba*

Morphologic characterization of the cyst form has been used for identification of the species within this genus, but variability in cyst structure can lead to some confusion in determining species (Stratford and Griffiths 1978). Clinical isolates often have very similar morphologies, and are thus difficult to identify. As attempts to classify *Acanthamoeba* using their structures have failed, deoxyribonucleic acid (DNA) -based techniques have arisen. For instance, whole cell or mitochondrial DNA has been purified, and the restriction fragment length polymorphism (RFLP) technique has been applied to identify clinical isolates (Kilvington et al. 1991; Kong et al. 2002). Recently, sequencing of nuclear small-subunit (SSU) ribosomal ribonucleic acid (rRNA) has given rise to genotypic distinction of 16 different clades of *Acanthamoeba* (T1 through T16 genotypes) (Corsaro and Venditti 2010). For field and clinical identification, polymerase chain reaction (PCR) amplimers from the 18S ribosomal DNA region have been identified that are specific for *Acanthamoeba* and are present in all genotypes; this amplimer is roughly 450 base pairs in size and is called ASA.S1. Due to lack of sufficient variability of this region for genotyping within this genus, a separate amplimer is sequenced and used (GTSA.B1) for identifying the specific clade of a specimen (Schroeder et al. 2001; Corsaro and Venditti 2010). The current phylogeny based on the entire 18S rDNA sequence is demonstrated in Figure 2.

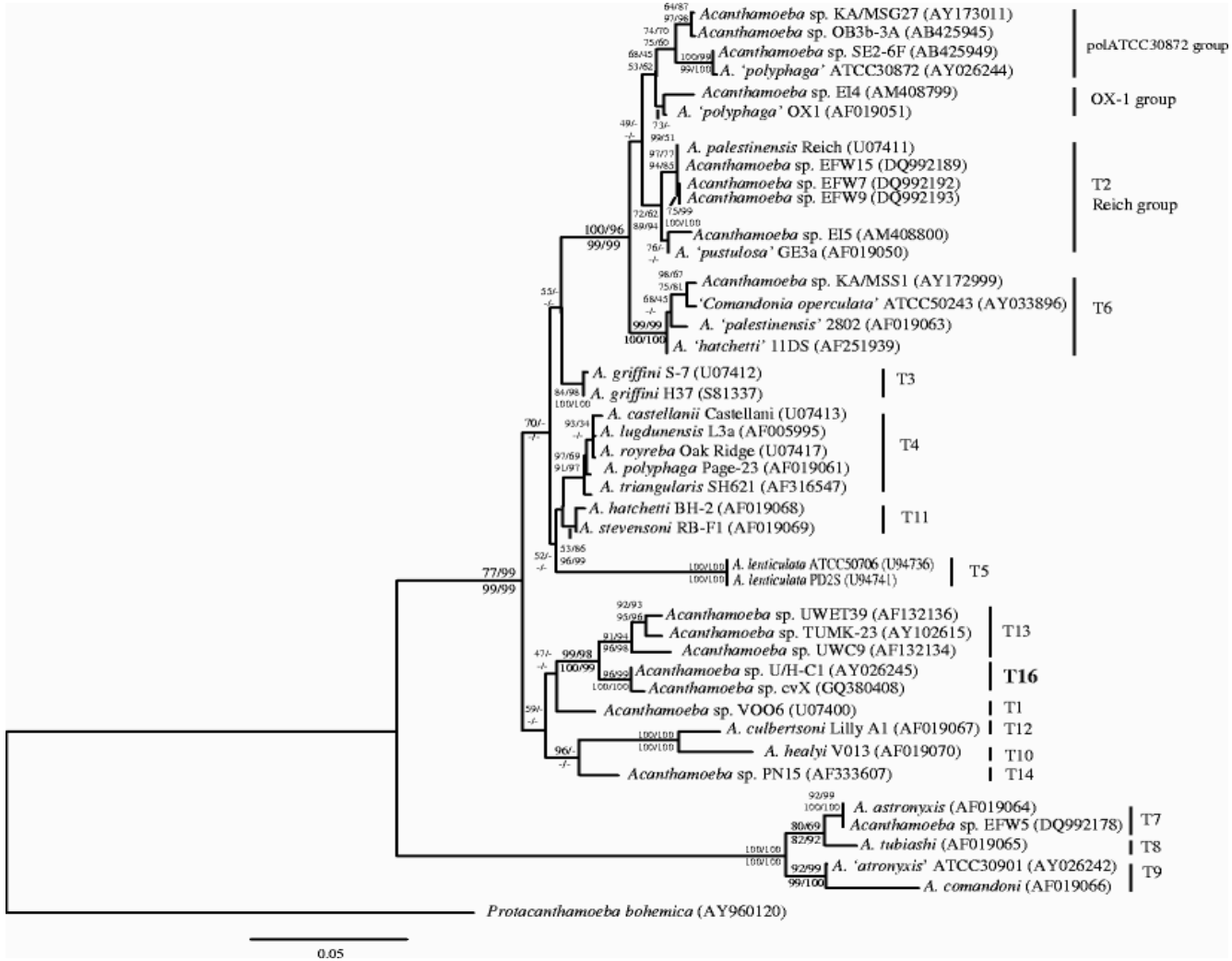

Figure 2. Current phylogenetic diagram of *Acanthamoeba spp*. and genotypes based on 18S rDNA sequencing done by Corsaro and Venditti (2010).

While these methods are sufficiently accurate to classify *Acanthamoeba*, their limitation is in the amount of time it takes to perform all of the steps necessary to positively identify isolates. Thus, sequencing-based methods are unlikely to be employed in a clinical setting.

Encystment

When confronted with stress conditions, such as starvation or changes in osmolarity, *Acanthamoeba* can form cyst structures (Cordingley et al. 1996) that are relatively dormant but preserve viability until more favorable conditions occur (Martinez 1985). Cyst wall formation involves the development of two distinct layers, the endocyst and the exocyst, separated by a space. The space between the cell walls is interrupted by points at which the walls join, called opercula; it is at these points that the excystment process begins. The inner cyst wall is mostly cellulosic, as determined by radio-labeled glucose incorporation (Weisman 1976), while the outer cyst wall is a discontinuous layer of protein and polysaccharides (Barrett and Alexander 1977) with an acid-resistant nature. Specific proteins present in the exocyst wall have been identified (Rubin et al. 1976; Hirukawa et al. 1998). The total chemical composition of the cyst walls combined is roughly a third cellulose, a third protein, five percent lipid, eight percent mineral, and the remainder unidentified (Neff and Neff 1969). Weisman (1976) described three distinct stages of the encystment process based on morphologic observations: induction, wall synthesis, and dormancy.

While all cyst wall structures have not been entirely elucidated, biochemical comparison of troph and cyst carbohydrate composition reveals a shift in metabolism

during the progression of encystment. For instance, the trophozoite carbohydrate profile is made up of roughly 98% glucose; in contrast, carbohydrates in cysts are composed of both glucose and galactose. The cellulosic component of cysts is roughly 55% glucose, 13% galactose, and 32% mannose (Dudley et al. 2009). Some distinction in structure and resistance has been observed between those cysts allowed to form over longer periods of time in growth medium and those that are forced to encyst using salt solutions (Bowers and Korn 1969; Hughes et al. 2003), and different species and genotypes have shown variability in chemical composition (Barrett and Alexander 1977). Furthermore, a great deal of species variability exists in biocide resistance (McBride et al. 2005). As much of the biocide resistance can be attributed to encystment, it follows that there is some species variation in the encystment process. While *A. castellanii* is generally used as a model organism, it might be prudent to examine the encystation process in other species as well.

For the purposes of investigation, it is possible to induce the semi-synchronous formation of cysts by exposing *Acanthamoeba* trophozoites to an encystment medium, such as the ones described by Neff et al. (1964), McMillen et al. (1974), or Hirukawa et al. (1998). Comparative analysis of different encystment media and strains reveals that the success of cyst induction depends on both the age of the isolate (and thus the period of time through which it has been axenically cultured) and the choice of encystment media, wherein alkaline media have been demonstrated to be most successful in inducing cyst formation (Kohsler et al. 2008). Also, subtle differences are observed in the way that each strain responds to the different media, or between members of the same species

and strain that are cultivated under varied conditions such as temperature, media, shaking, etc. (Buck et al. 2000). These differences might give rise to variations in resistance to challenge; for example, Hughes et al. (2003) note varying sensitivity to contact lens solutions in *A. polyphaga* cysts produced through different methods. Further, variation in methods used to test the efficacy of the solutions prevents adequate comparison of previous studies (Buck et al. 2000).

Cellular events that give rise to the formation of the cyst wall result from a metabolic shift, such as in the synthesis of cellulose for the cyst wall (Weisman 1976). These changes should be evident and demonstrable through examination of genetic regulation. Bowers and Korn (1969) described visible changes during encystment in the mitochondria, Golgi, and vacuoles, as well as changes in the lipid fractions of the cell. Furthermore, the process of cyst formation occurs through a distinct set of stages; for instance, the first notable change upon the beginning of encystation is the rounding of the cell (Neff et al. 1964).

Pathogenicity

The trophozoite stage of *Acanthamoeba* is capable of opportunistic infection, as seen in the case of granulomatous amoebic encephalitis (GAE), a very rare but life-threatening infection of the brain. It is generally known, however, as a potential infectious agent of the eye; this organism can cause keratitis, even in immunocompetent individuals (Marciano-Cabral and Carbral 2003). The first case of *Acanthamoeba* keratitis (AK) reported in the United States occurred in 1973, established in the eye of a rancher after significant eye trauma (Stehr-Green et al. 1989). However, AK in the

industrialized world generally occurs in conjunction with the use of contact lenses, and the relationship has clearly been established, as more than 80% of the cases of AK occur in contact lens wearers (Massachusetts Medical Society 1986). The use of cistern or grey water in cleaning of lenses or in preparation of contact lens solutions has been found to contribute to the incidence of keratitis caused by amoeba, as these water sources are suspected harbors for *Acanthamoeba* (Kilvington 1990). Many of the cases of AK have been linked to improper cleaning of the lenses or cases, and their contamination with both bacteria and amoeba (Stehr-Green et al. 1987). Furthermore, as determined by a case-controlled study, use of particular lens cleaning solution (AMO Complete MoisturePlus Multi-Purpose Solution) with soft lenses is associated with AK (Joslin et al. 2007). Roughly one in 250,000 contact lens wearers is diagnosed with keratitis cause by *Acanthamoeba*; while the incidence is relatively low, the infection itself is serious, as it is painful, difficult to treat, and can lead to the loss of eyesight (Stehr-Green et al. 1989). In addition to the use of contact lenses, there are other important factors contributing to the incidence of AK. In less developed countries, such as India, corneal trauma is more of a risk factor than contact lens use (Sharma et al. 2000).

The pathogenicity of the *Acanthamoeba* involves a complex series of events that is initially facilitated by the presence of a mannose-binding glycoprotein (MBP) on the surface of the cell membrane (Garate et al. 2005). The MBP is roughly 400 kilodaltons (kDa) in size, spans the membrane (Garate et al. 2004), and has a 136 kDa region with mannose affinity on the surface of the membrane (Yang et al. 1997). Interestingly, corneal epithelium that is damaged often shows increased expression of mannose

glycoproteins; this helps to explain the increased risk of AK in the case of corneal abrasion or other trauma (Jaison et al. 1998). After adhesion is facilitated by the binding of the MBP to the mannose glycoproteins on the surface of the host cells, the amoeba induce the cytopathic effect (CPE), or the disruption of the epithelium and ultimately the penetrance into the stroma. The destruction of the epithelial layer of cells is caused by the induction of mannose induced protein (MIPs), such as MIP133 and other cytopathic factors, which act to cause cell lysis (Clarke and Niederkorn 2006). After they reach the stroma, *Acanthamoeba* are reliant on proteases that break down collagen, as well as plasminogen activators that cause the host to release proteolytic enzymes such as matrix metalloproteinases; this leads ultimately to cell death, as illustrated in Figure 3 (Larkin et al. 1991; Clarke and Niederkorn 2006).

The genetic-based classification of *Acanthamoeba* strains has revealed that the majority of disease-causing strains are of the T4 genotype sequence (Schroeder et al. 2001; Walochnik et al. 2000; Booton et al. 2002; Zhao et al. 2010). By morphologic classification, *A. castellanii* and *A. polyphaga* are the most common of the amoebic species that cause keratitis (Dart et al. 2009).

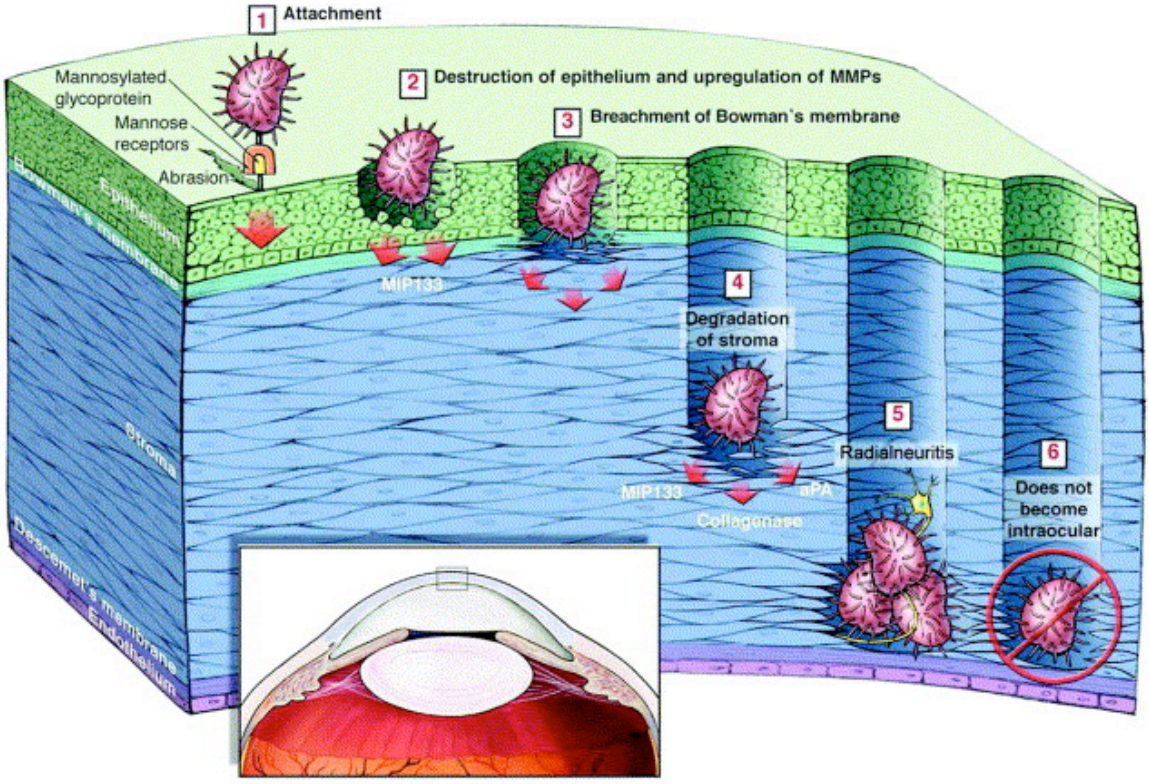

Figure 3. "The pathogenic cascade of *Acanthamoeba* keratitis." This illustrates the steps involved in the cytopathic effect and elucidates the mechanisms by which *Acanthamoeba* destroy the infected cornea in AK (Clarke and Niederkorn 2006).

Encystation is clinically relevant because once the cyst wall has formed, the amoeba are difficult to treat with antibiotics (McClellan et al. 2002; Lloyd et al. 2001). The physical barrier provided by the double cyst wall becomes more effective throughout the encystment process, rendering the amoeba protected from many biocides (Turner et al. 2006). Since they are relatively dormant, the cysts themselves are most likely incapable of causing infection (Dudley et al. 2005), but can establish pathogenicity and cause CPE upon excystation. Furthermore, cysts can excyst more than 20 years after formation and emerge as viable pathogens (Mazur et al. 1995; Sriram et al. 2008). In the specific context of potential keratitis, some chemicals in contact lens solutions have been shown to cause encystation of trophozoites that are present (Lonnen et al. 2010). The combination of these chemicals with water treatment procedures that leave *Acanthamoeba* trophozoites in the water has been the hypothesized cause of recent *Acanthamoeba* keratitis outbreaks in the United States (Cohen 2009). Prevention of encystation might be considered a reasonable route to blocking the pathogenicity of amoeba, and advances in an understanding of the genetics of encystation would be useful (Makioka et al. 2000; Makioka et al. 2001).

Current treatment options for AK are limited, and there are not drugs specifically licensed for AK treatment. Infected patients are treated topically with biguanides, such as polyhexamethylene biguanide or chlorhexidine, sometimes combined with diamidines such as propamidine isethionate or hexamidine. Additionally, topical steroids are applied in order to reduce inflammatory damage to the epithelial tissue (Dart et al. 2009). Recent explorations of naturally occurring compounds, such as sesquiterpene and lapachol, are

promising, but to date have only been used to challenge the trophozoite form of *Acanthamoeba* (Martin-Navarro et al. 2010). However, it is widely accepted that cysticidal drugs are necessary for effective AK treatment, since the trophozoites have the capability of encystment to avoid chemical assault (Awwad et al. 2007).

Prognosis is generally poor for patients that are not diagnosed and treated relatively quickly, as persistent and recurrent infection occurs in most patients who are not treated within the first 3 weeks of infection. Presumably, this time period allows the formation of many viable cysts that are then very difficult to kill (Dart et al. 2009); patients that are diagnosed late (after three weeks from the date of infection) often require corneal transplant or even enucleation (Tu et al. 2008).

Acanthamoeba as reservoirs

In addition to causing disease, *Acanthamoeba* can serve as reservoirs for other pathogenic bacteria that are able to divide or simply survive within the cell, such as *Pseudomonas, Legionella,* or *Mycobacterium* (Brown and Barker 1999; Greub and Rauolt 2004). *Francisella tularensis*, the causative agent of tularemia in humans, is also harbored within amoeba (Abd et al. 2003). Some scientists have even suggested that survival inside *Acanthamoeba* hosts provides an evolutionary "training ground" by which pathogens may further refine their abilities to survive within a human host (Harb et al. 2000). Price et al. (2009) demonstrated that intracellular pathogens, such as *Legionella*, exploit pathways that are highly conserved and thus similar in both the protozoa and human macrophages, giving credence to this suggestion. Furthermore, Cirillo et al. have demonstrated increased virulence in *Legionella pneumophila* (1999) and *Mycobacterium*

avium (1997) when passed through *A. castellanii*. As this hypothesis has gained wider acceptance, growth in *Acanthamoeba* has been used as a criterion for screening of environmental isolates to detect potential pathogens (Furuhata et al. 2009).

Even though pseudomonads seem to have an inhibitory effect on amoeba at high concentrations (Qureshi et al. 1993; Wang and Ahearn 1997), the two can be found together in some contact lens cases (Donzis et al. 1989). Cyst formation is quickly stimulated in those amoeba grown concurrently with live *Pseudomonas aeruginosa* (Wang and Ahearn 1997). The interactions between the phagocytic amoeba and their potential bacterial targets are complex, and influenced by a number of factors including amoeba preference for some as food, specific recognition and binding proteins on the surface of the amoeba, and the beneficial or toxic effects of the different bacterial species (Morales and Alfieri 2008). It is unclear how the co-culture of bacteria and amoeba may influence the regulation of genes involved in cyst formation. Further, the ubiquity of *Acanthamoeba* species in the natural environment means that they might provide a previously unexplored reservoir for several potential pathogens. While the detection of pathogens within amoeba is somewhat complicated, there have been successful attempts to examine both species using flow cytometry and through isolation and distinction of total genomic DNA of a sample (Schmitz-Esser et al. 2008; Heinz et al. 2007).

Interestingly, it has also been suggested that the expression of mannose on the cell wall of bacteria such as *Corynebacterium xerosis*, natural flora of the eye, might enhance virulence of *Acanthamoeba* in their potential to cause keratitis (Alizadeh et al. 2005). For these reasons, it might be of particular interest to examine cross talk between species.

Determining viability

Detecting viability in cysts presents a particular challenge in the laboratory, as they are relatively resilient, and do not usually lyse or appear different microscopically when they are not metabolically active. Also, many stains that might assist in determining viability are often difficult to get into the cell, as the cyst wall structure is very resistant to penetrance of dead-selective dyes such as propidium iodide (PI). One technique that may be used in industry to determine viability after challenge with a biocide involves using a soft agar containing bacteria, plating of the challenged amoeba, and then looking for the clearing of the bacteria as an indicator of excystment and viability (Hugo et al. 1991). However, drawbacks to this technique include an inability to determine precise numbers (as a single clearing does not necessarily indicate a single viable amoeba), and the length of time that it takes to run this assay (days to weeks).

Encystment Genes

Through examining changes that occur during encystment, "early" and "mature" cysts are distinguishable and could be comparatively studied in terms of their relative metabolic and genetic properties. An understanding of the proteins and their genetic regulation during the encystment process would be invaluable to the development of potential therapeutic targets. Specific genes that are regulated in association with these cellular changes can be identified. For instance, messenger ribonucleic acid (mRNA) screening has been performed to compare gene expression in trophozoites to that in cysts to identify cyst-specific genes; generation of expression sequence tags (ESTs) combined with data mining reveals a complete picture of those genes differentially expressed during

encystation and has identified 23 genes that are expressed only in the cysts (Moon et al. 2008a). As more than half of the genome of *A. castellanii* has been sequenced to date (Anderson et al. 2005), and an EST database for virulence and stress conditions has been established (Moon et al. 2009), there is an available platform for further molecular exploration.

In addition, electrophoretic separation and comparison of cyst and trophozoite proteins has been used to identify and characterize those which are specific to the cyst, such as CSP21, which is a hydrophilic cyst wall protein that is transcribed and translated only in encysting cells (Hirukawa et al. 1998). Leitsch et al. (2010) recently used a similar proteomics approach to determine that most of the changes in protein profile associated with encystment occur during the first 24 hours of the process.

One example of an "encystment gene" is a serine protease, called encystment mediating serine protease (EMSP), characterized by Moon et al. (2008b); they suggest it is involved in autolysis for bio-molecule recycling during the encystation process. It has been demonstrated that the activity of serine protease is necessary for encystment to take place; using serine protease inhibitors such as phenylmethylsulphonyl fluoride, it is possible to block both encystment and excystment (Dudley et al. 2008). Furthermore, Lorenzo-Morales et al. (2005) recently demonstrated the ability to block encystation using a siRNA complementary to the serine protease gene. Similarly, the role of cysteine proteases was recently elucidated, but these are present in the trophozoite form of *Acanthamoeba* as well as the cyst form. Early protein turnover during encystment,

therefore, is likely to rely on proteases already present in the trophozoite, such as cysteine proteases, but cyst-specific proteases are also necessary (Leitsch et al. 2010).

From a regulatory standpoint, induction of encystment has only recently come under examination. The use of monoclonal antibody to a trophozoite surface protein induces encystment and prevents excystment, suggesting that the processes of differentiation are, at least to some extent, under control of surface receptors (Yang and Villemez 1994). The use of inhibitors has demonstrated that the encystment process is partly dependent on tyrosine kinase-mediated signaling. The Ras protein - cyclic adenosine monophosphate (cAMP) pathway, previously studied in yeast, is involved; the mitogen-activated protein kinase (MAPK) pathway is likely not involved. Additionally, actin polymerization is necessary for proper cyst formation, as cytoskeletal rearrangement must take place (Dudley et al. 2009). Furthermore, 21 histidine kinases have been predicted through sequencing, potentially playing a role in allowing *Acanthamoeba* to sense and respond to many different signals in the environment, including stress conditions (Anderson et al. 2005).

Glycogen phosphorylase also plays a role in encystment, presumably liberating simple sugars for the construction of the cellulosic component of the cell wall. It has been long known that glycogen levels decrease during the encystment process (Weisman et al. 1970), but the mechanism by which this occurred during early encystment was unknown until relatively recently. Lorenzo-Morales et al. (2008) used a Western blotting technique to demonstrate that glycogen phosphorylase expression occurs only within the first 24 hours of encystment; cells that underwent glycogen phosphorylase RNA

knockdown were unable to form double-walled cysts. In addition to glycogen phosphorylase, cellulose synthase is likely involved in creation of the endocyst wall. Sequencing studies have revealed some similarities with *D. discoideum* cellulose synthase genes (Anderson et al. 2005). Understanding of the cellulose synthetic pathway in *Acanthamoeba* might provide the basis for therapeutic targeting of cellulose synthesis (Dudley et al. 2007).

Comprehensively, these studies are indicative of the shift in gene expression that occurs during *Acanthamoeba* encystment. These genes may serve as targets for differential gene expression assays using reverse transcription coupled with polymerase chain reaction (RT-PCR).

RT-PCR

RT-PCR has been used to study the encystment process. For instance, Moon et al. (2009) have studied a protein involved in autophagy, ATG8, using this technique. ATG8 is bound to the autophagosome membrane, and is up regulated during encystment, with levels of expression increasing between three- and four-fold in encysting Acanthamoeba after three days. In this way, the timing of cellular events that give rise to the mature cyst structure can be determined; this can be extremely useful in the eventual development of treatment protocols in the case of AK or other *Acanthamoeba* infections.

1.2 MATERIALS AND METHODS

Culture methods for *Acanthamoeba* trophozoites

Acanthamoeba castellanii (ATCC 30234) and *Acanthamoeba polyphaga* (ATCC 30461) were grown in proteose peptone-yeast extract-glucose (PYG, 2% proteose peptone, 0.1% yeast extract, 4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.05 mM $Fe(NH_4)_2(SO_4)_2$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 0.1% sodium citrate dihydrate, and 0.1 M glucose, pH 6.5) broth axenically at 25°C on a shaker set to 40 revolutions per minute (rpm). Cultures were maintained in 125 mL of broth in 250 mL Erlenmeyer flasks with foam stoppers, and sub-cultured every other week; unless otherwise specified, 10 day-old cultures were used for all DNA extractions and inoculation of encystment medium unless otherwise specified.

Cell Imaging

All cellular images were obtained using the QImaging MicroPublisher 3.3 RTV (Surrey, British Colombia, Canada) attached to a Nikon Optiphot-2 (Melville, NY) at 200X or 400X magnification.

Encystment assays

"Natural cysts" were created by plating cells onto non-nutrient agar (NNA) plates, made with 1.5% Bacto agar (Becton Dickinson, Sparks, MD) in distilled water, as originally described by Dudley et al. in 2009. Cells from 14 day-old cultures were centrifuged at 3,000 relative centrifugal force (rcf) in a microcentrifuge for 10 minutes, and the supernatant was removed. Cells were re-suspended in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) to a final

concentration of 1.0 x10^6 cells per mL. This suspension was then used to inoculate the NNA plates (2.0 mL per plate), and spread with a flame-sterilized glass hockey stick. Plates were then stored at room temperature until cysts were harvested.

Various liquid encystment media were also used to induce encystment (Table 1). For each of these, trophs of 10 day-old cultures were centrifuged at 3,000 rcf for 10 minutes, and supernatant PYG was removed. These cells were then re-suspended in the encystment media to a final concentration of approximately 10^5 cells per mL in 50mL conical tubes, and incubated at 30°C for 1 week.

Table 1. Encystment media used in this study.

Encystment Media	Medium Components	Reference
Hyperosmotic Glucose	8% (by weight) glucose in PBS	Dudley et al. 2005
TRIS (Alkaline)	95mM NaCl, 5 mM KCl, 8mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$, 20 mM Tris-HCl, pH 9.0	Hirukawa et al. 1998
McMillen's Medium	in 1 L water: 7g NaCl, 3 mg $MgCl_2$, 142 mg Na_2HPO_4, 136 mg KH_2PO_4, 3 mg $CaCl_2$, 3 mg $FeSO_4$, pH unadjusted	McMillen et al. 1974
Taurine	1/4 strength Ringer's solution, 20 mM taurine, 15 mM $MgCl_2$ Ringer's Solution: 8.6 g/L NaCl, 0.3 g/L KCl, 0.33 g/L $CaCl_2$	Hughes et al. 2003

Encystment rates were determined by removing aliquots of cells for each media over time: 6 hours, 1 day, 2 days, 3 days, 4 days, and 5 days. An additional aliquot was taken for *A. polyphaga* at 7 days. Each aliquot was centrifuged at 3,000 rcf for 10 minutes, and then the supernatant encystment medium was decanted. Afterwards, each pellet was re-suspended in 500µL of PBS. After vortexing, total cell numbers were

counted in triplicate or quadruplet using a Hausser Scientific hemocytometer (Horsham, PA) and cell counts were then averaged. Cells were then treated with final concentration of 0.25% sodium dodecyl sulfate (SDS), using 1.25 μL of 10% stock SDS solution in 500 μL of cyst suspension, and allowed to incubate at room temperature for 30 minutes. Cell populations were then re-counted in quadruplicate and averaged, and total cyst percentage was determined with the following calculation: (average # of cells present after SDS treatment / average # of cells present before SDS treatment) x 100 = % cysts.

Determining viability of cysts

To determine viability of the cysts produced by each method, 10 mL of cells suspended in encystment media for 7 days was centrifuged at 3000 rcf for 10 minutes. Supernatant encystment media was discarded, and the pellet was re-suspended in 1.0 mL PBS. Cells were counted using the hemocytometer twice, and the average value for the two counts was recorded. After counting, 2.5 μL of 10% SDS was added to each suspension, and vortexed. Cells were allowed to incubate at room temperature for 30 minutes, at which point they were re-counted using the hemocytometer. Average values of 4 counts were recorded, and cyst percentage of the total population was calculated.

To remove SDS before plating, cells were once again centrifuged at 3,000 rcf for 10 minutes. Supernatant was removed and an additional 1.0 mL of PBS was added to each pellet and vortexed for wash purposes. Cells were centrifuged at 3,000 rcf for 10 minutes, and 200 μL of PBS was added to the pellets for re-suspension. Cell counts were once again performed using a hemocytometer, and the average value of 2 counts was

recorded. At this point, 1:10 dilutions and 1:100 dilutions were made of each cell suspensions in PBS.

For each dilution, triplicate viability assay plates were set up as follows. To make the plaque assay plates, non-nutrient agar (1.5% agar by weight) was poured into sterile petri plates. This was allowed to solidify before the soft agar overlay was added. The soft agar overlay was made using sterile 0.5% agar with heat-killed *Enterobacter aerogenes*. The *Enterobacter* was inoculated into 500 mL of tripticase soy broth (TSB) and allowed to grow overnight at 30°C. The next day, the *Enterobacter* was centrifuged at 3,000 rcf in 50 mL Falcon tubes. The supernatant was removed, and the pellets were combined; the combined pellet was then heat-killed by boiling for 10 minutes. Once this heat-killed *Enterobacter* was added to the soft agar, a thin layer was poured over the non-nutrient agar plates, and allowed to solidify.

These plaque assay plates were then used to determine viability. Five 10 μL aliquots from each dilution were spotted onto the plates, in triplicate. They were allowed to sit at room temperature for four days before examination for clearings. The number of clearings was counted for each plate, and a most probable number (MPN) table was used to determine the probable number of viable cells in each 10 μL aliquot. The percentage viability of cysts for each encystment method was then calculated using the following formula: (number of viable cells x dilution factor / number of cells in hemocytometer count prior to spotting on plates) x 100.

Harvesting of cysts from NNA plate

Cyst harvesting from NNA plates (in triplicate) occurred at the following time points after NNA inoculation: 6 hours, 1 day, 2 days, 3 days, 4 days, and 5 days. 2.0 mL of sterile PBS was added to each NNA plate, and the plate was gently scraped with a flame-sterilized glass hockey stick. All liquid was collected, and this process was repeated. The cells were centrifuged at 3,000 rcf for 10 minutes, and supernatant was decanted. The cell pellet was then used for RNA extraction. Similarly, cysts were harvested from liquid encystment media at the following time points after inoculation: 6 hours, 1 day, 2 days, 3 days, 4 days, and 5 days. For each time point, 10 mL of media containing cysts was centrifuged at 3,000 rcf for 10 minutes, and the supernatant media was decanted. The pellet was then used for RNA extraction.

Nucleic Acid extraction

DNA extraction was carried out using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). Standard protocol for cultured cells was followed using 1.0×10^6 cells in the spin columns with the provided reagents and buffers. DNA concentration was measured through spectrophotometry at 260 nm, using the NanoDrop 1000 Spectrophotometer (Wilmington, DE).

RNA extraction was done with TRIzol Reagent (Invitrogen, Carlsbad, CA), a monophasic solution of guanidine isothiocyanate and phenol, according to manufacturer's protocol. A second purification was done with all of the samples using the Illustra RNAspin mini kit (GE Healthcare, Buckinghamshire, UK) according to protocol supplied with the kit; steps for tissue homogenization were skipped. Samples

were dissolved in 0.1% diethylpyrocarbonate (DEPC) (Sigma, St. Louis, MO) in water, and RNA was quantified after each purification step using the NanoDrop spectrophotometer. All RNA samples were screened using PCR for DNA contamination, and checked for integrity using RT-PCR with primers targeting 18S RNA (Figure 4).

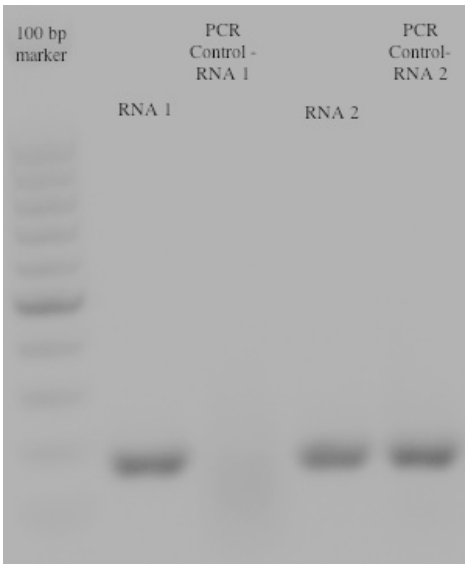

Figure 4. Example of AGE image of screening reactions for two different RNA samples. RNA 1 and RNA 2 lanes each show bands representing RT-PCR reactions that were positive for 18S RNA; the PCR control, however, shows DNA contamination for the RNA 2 sample.

Primer Design

The primer library (Tables 2-4) was developed to target potential encystment and reference genes. Primers were all designed using Integrated DNA Technologies (Coralville, IO) software PrimerQuest available on their website (www.idtdna.com), and then purchased from IDT. Sequences used for primer design were obtained from European Molecular Biology Laboratory (EMBL) database sequences, GenBank sequences, published literature, or EST data provided by the Moon group. In general, all target sequences were examined using the protein tool on the National Center for

Biotechnology Information (NCBI) website in order to limit primer design to conserved regions. Candidate reference genes were chosen based on Bohle et al.'s (2007) attempt to select reference genes for *Aspergillus niger*, and through scanning the annotated genome of *Acanthamoeba castellanii* for similar targets.

Upon receipt, primers were reconstituted with autoclave and filter-sterilized de-ionized water (diH_2O) to a concentration of 100 μM. Working stocks of 10 μM primers were made, and all were stored at -20°C. All primers were then screened using both *A. castellanii* and *A. polyphaga* template DNA with PCR.

Table 2. Primers used in this study to target the encystment genes glycogen phosphorylase and serine protease. All primers were designed using the IDT tool PrimerQuest, available on their website (www.idtdna.com).

Primer Set	Forward Primer Sequence	Reverse Primer Sequence	Reference
Glycogen Phosphorylase Primers			
8399/8400	TGATCCAGGTGGGCTACAAGAAGT	AGACGAAGAGGAACTGCTGCTTGA	Designed using reverse complement of EMBL accession no. EC109277
2156/2157	AAGCTGGAGGACCTCTACGA	TGCTTCTACGACAGCTGCGCGTA	Lorenzo-Morales et al. 2008
0764/0765	TACCCGAACGACCACCACTACAA	TAGAGCTCCGAGGTTCTTGCCAAA	Designed using reverse complement of EMBL accession no. EC109277
5532/5533	AATTCGGCACGAGGATGAAGTTCG	AAGCGCTTGTCCATCTTGAGCGA	Designed using EST sequence provided by Dr. Moon
7788/7789	CGACGAGTCAATTGAGCATTTGTTG	AGACGCTTCAGTACCAGCAGTAGA	Designed using conserved regions of glycogen phosphorylases
Serine Protease Primers			
2150/2151	ACTTCAAAGGCAACTACACCCAGGA	AGATGGTGTTGTAGGCGCTGTT	Designed using GenBank accession no. EU365402
5668/5669	ATGTCCCTCGGCGGCGGCTAC	CATGGAGGTGCCGGAGATGGT	Designed using conserved regions of serine proteinases
7328/7329	TCATGTCGGTACCGTCGGTGG	AGTTGGAGAACGAGGCGCGGAT	Designed using conserved regions of serine proteinases
5530/5531	CAACAAGATGCTCTACACGCC	CGTAATCGGAGCCATCCAACA	Moon et al. 2008b

Table 3. Primers used in this study to target the encystment genes enolase and cellulose synthase. All primers were designed using the IDT tool PrimerQuest, available on their website (www.idtdna.com).

Primer Set	Forward Primer Sequence	Reverse Primer Sequence	Reference
Enolase Primers			
5534/5535	ATCAAGGCGTGCCTGGTCT	GATCCTGAGGAGCTGGTTGTACTT	Designed using EST sequence provided by Dr. Moon
4770/4771 (outward)	TCGGCAAGTACAACCAGCTCCTCA	GCTCCTGTGCGACACCATCA	Designed using sequenced amplicon from 5534/5535
7330/7331	ACTGCTGAAGTCTGCCATCGAGAA	TTCCCACCACGGTGAAACTCTGAT	Designed using GenBank Accession no. DQ487988.1
5200/5201	TGCAGCAACAATCCATGCCAATCCTC	TCATCCTGCCTGTTCCTGCCTTTA	Designed using reverse complement of GenBank Accession no. DQ497988
5202/5203	GCTGCAAACTGGAACAAGCAGACA	ACTGCTGAAGTCTGCCATCGAGAA	Designed using reverse complement of GenBank Accession no. DQ497988
Cellulose Synthase Primers			
2152/2153	CGTGAATCAAGAGGTTGGGCACAT	TGGGTGGTTTACGTCTACGAAGGT	Designed using GenBank Accession no. AF163835
2154/2155	ATGGTGGTAGTGGTGACGATGGTA	AACCGATTGAAGATGTGCCCAACC	Designed using GenBank Accession no. AF163835
0766/0767	CCGTTCACAATATTCGTCCACCTG	TCAGCATCATCGGCATCTCTTGGA	Designed using GenBank Accession no. AAF00200
0768/0759	CGTGAATCAAGAGGTTGGGCACAT	CAGGTGGACGAATATTGTGAACGG	Designed using GenBank Accession no. AAF00200

Table 4. Primers used in this study to target potential reference genes, including 18S ribosomal DNA and others. All primers were designed using the IDT tool PrimerQuest, available on their website (www.idtdna.com).

Primer Set	Forward Primer Sequence	Reverse Primer Sequence	Reference
18S ribosomal DNA primers			
4772/4773	TGCCACCGAATACATTAGCATGGG	AACATCCTTGGCAGATGCTTTCGC	Designed using GenBank Accession no. U07413.1
1508/1509	GAGTGTTCAAAGCAGGCAGATCCA	CTAGCGCGGCATATTTAGCAGGTT	Designed using GenBank Accession no. U07413.1
Other potential reference gene primers			
9456/9457 (Myosin)	AAGAACTACTCCTCGGTCGACAAC	TTCTCGCCCATGTTGCACAGGAT	Designed using GenBank Accession no. U67860.1
9458/9459 (Cytochrome oxidase I)	ATGCCGGTTATGATAGGCGGTTTC	GCTACAATAGAACTTAACGGCGGG	Designed using NCBI reference sequence NP_042527.1
9460/9461 (Cytochrome oxidase II)	TGCTGTTCCATCATTTGCTCTACT	CCCATGCCCTTGTACATCATTTCC	Designed using NCBI reference sequence NP_042527.1
9462/9463 (Cytochrome oxidase III)	GTTTGGTGAAGAGACGTGATTCGTG	AGGTGGCCAAACTGAACCTAACAC	Designed using GenBank Accession no. U12386.1
9464/9465 (ATPase)	CCTAGTAAATGACAATATGCGGTGG	GGCCTGTAATAGTAAATCCATAGGGT	Designed using NCBI reference sequence NC_001637
9466/9467 (Profilin)	TGTCATCACCGTCAAGACCTCGAA	AGGGAAGAAAGAGTGGGTACAAAGG	Designed using Nucleotide Accession no. AF414696

Polymerase Chain Reaction

For each PCR reaction, Taq polymerase Master Mix (Qiagen, Germantown, MD) containing deoxynucleotide triphosphates (dNTP's), buffer, and Taq polymerase was combined with 10-100 ng of template DNA (depending on the reaction), 1.0 μL each of 10 μM forward and reverse primers, and enough autoclave and filter sterilized diH_2O to bring the reaction volume to 25.0 μL in 0.2 mL thin-walled PCR tubes. Reactions were then placed in an Eppendorf Mastercycler Gradient (Hauppauge, New York), with an initial 10 minute 94°C dissociation step, followed by 30-45 amplification cycles (94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 1 minute) and then a 10 minute 72°C final extension step; the reaction was held at 4°C until removed from the thermal cycler. The PCR product was stored at -20C for future sequencing, or immediately loaded into a 1.2% agarose gel with the DNA-binding fluorescent dye Biotium Red (Biotium, Hayward, CA) along with a 100bp low-scale DNA Ladder (Fischer Scientific). Agarose gel electrophoresis (AGE) was performed with power set to 90 volts (V) for 100 minutes; the gel was imaged using Foto/Analyst PC Image Software interface with the Investigator/Eclipse workstation (Fotodyne, Hartland, WI).

DNA Purification

DNA was excised and then purified from agarose gels using QIAquick PCR purification kit (Qiagen, Germantown, MD); the standard gel extraction protocol recommended by the manufacturer was followed. The gel was imaged before and after excision to confirm amplicon removal (see Figure 5, for example). All samples were quantified using the NanoDrop spectrophotometer.

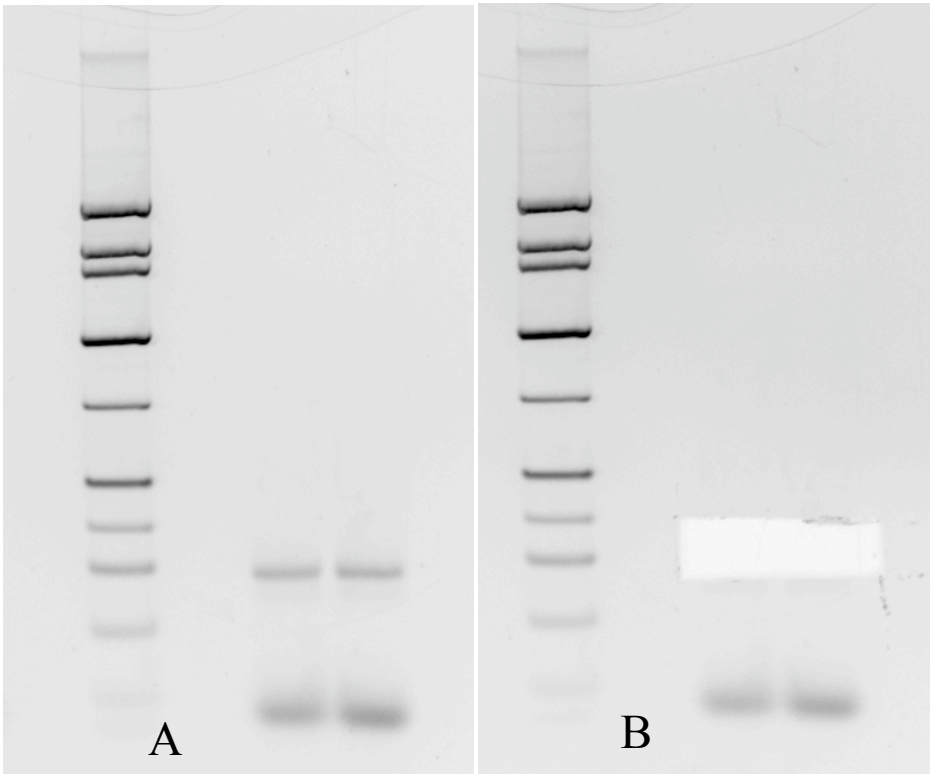

Figure 5. AGE image of before (A) and after (B) excision of ~300bp amplicon. PCR was performed using primers targeting the subtilisin-like serine proteinase (SLSP) and *A. castellanii* genomic DNA. The excised amplicon was purified using the Gel Purification Kit (Qiagen) and then sequenced to confirm target.

DNA Sequencing and Analysis

DNA purified from the gels was sequenced in a 3100 Genetic Analyzer (Applied Biosystems). Sample preparations were done to allow for 10-20 ng of template per 200 base pairs (bp) of fragment length, 1 μL of 10 μM primer, and sterile water to a total of 28 μL volume. Once sequences were obtained, analysis of sequence quality was done using 4Peaks 1.7.1 software (Mekentosj, Aalsmeer, The Netherlands). Low quality tail sequences were removed and alignment of forward and reverse complement of reverse sequences was completed with the Basic Local Alignment Search Tool (BLAST) (Nucleotide Blast, NCBI) available on the NCBI website. Furthermore, final sequences were BLASTed against databases to search for homology with published sequences.

RT-PCR

Reverse transcription was done using Superscript III One-Step RT-PCR kit (Invitrogen) using 10 ng of template RNA per reaction and according to the protocol supplied with the kit. Reverse primers from previously screened primer sets were used to generate gene-specific cDNA's (Table 5). The cDNA's generated were then used as template for a PCR reaction in the same tube, using Ampligold Taq enzyme supplied in the reaction mix. Each reaction was set up for a total reaction volume of 25 μL, with 0.5 μL of each 10 μM forward and reverse primer.

Table 5. Primers used in reverse transcription for cDNA synthesis.

Target Gene	Organism	Primer #	Primer Sequence
Serine Protease	*A. castellanii*	5531	CGTAATCGGAGCCATCCAACA
	A. polyphaga	2151	AGATGGTGTTGTAGGCGCTGTT
Glycogen Phosphorylase	*A. castellanii*	8400	TAGAGCTCCGAGGTTCTTGCCAAA
	A. polyphaga	8400	TAGAGCTCCGAGGTTCTTGCCAAA
Cellulose Synthase	*A. castellanii*	0759	CAGGTGGACGAATATTGTGAACGG
Enolase	*A. polyphaga*	5535	GATCCTGAGGAGCTGGTTGTACTT

Randomly Amplified Polymorphic DNA (RAPD) Typing

DNA was extracted from each strain type using the method described above. PCR was then carried out with six random ten base pair primers (Table 6), chosen among those that have previously worked to differentiate strains of the same species eukaryote, one primer in each reaction tube (Aufauvre-Brown et al. 1992).

Table 6. Primers used in RAPD typing reactions.

Primer Name	Primer Number	Primer Sequence
R106	1	CGTCTGCCCG
R108	2	GTATTGCCCT
R120	3	GAATTTCCCC
R128	4	GCATATTCCG
R141	5	ATCCTGTTCG
R151	6	GCTGTAGTGT

For each reaction tube, 12.5 µL Taq polymerase Master Mix was combined with 50 ng of template DNA, 1.0 µL of 10 µM primer, and enough autoclave and filter sterilized diH2O to bring the reaction volume to 25.0 µL. The reactions were then placed

in an Eppendorf thermal cycler, with an initial dissociation step at 94°C for 10 minutes, followed by 45 amplification cycles (94°C for 30 seconds, 35°C for 30 seconds, 72°C for 1 minute and 30 seconds) and then a 10 minute 72°C final extension step; the reaction was then held at 4°C until removed from the thermal cycler. The product was immediately loaded into a 1.2% agarose gel with Biotium Red, along with a 100bp Low-Scale DNA Ladder. Electrophoresis was performed at 90V for one hour, and the gel was then imaged.

1.3 RESULTS

Comparison of Encystment Methods

Reported levels of encystment in *Acanthamoeba* species vary greatly; *A. castellanii* and *A. polyphaga* encystment is reported to occur at vastly different rates by different groups working with these organisms. In order to determine whether this variation was due to the use of different encystment methods by various investigators, several different chemical media and the non-nutrient agar method of encystment were compared for their effect on encystment of *A. castellanii* (Table 7; Figure 6) and *A. polyphaga* (Table 8; Figure 7).

Table 7. Comparison of *A. castellanii* cysts created by various methods surviving 30 minute treatment with 0.25% SDS. Trophs from an axenic culture were taken at 10 days old and inoculated into various encystment media. 0.5 mL of cells were taken from the various encystment media, centrifuged at 3,000 rcf for 10 minutes, and supernatant was removed. All cells were re-suspended in 500 µL sterile PBS, and then counted both prior to addition of SDS and after 30 minute incubation with SDS. All numbers presented represent averages of three or four different counts.

Encystment Method		**Day 0** (# cells per µL)	**Day 1** (# cells per µL)	**Day 2** (# cells per µL)	**Day 3** (# cells per µL)	**Day 4** (# cells per µL)	**Day 5** (# cells per µL)
8% Glucose	pre-SDS	52	36	41	18	16.5	17
	post-SDS	0	0	0.5	1.5	1	0.75
TRIS	pre-SDS	52	58.5	37	36.5	41.5	32
	post-SDS	0	0	1.75	1.75	5	2.5
Taurine	pre-SDS	52	47.5	54.5	29.5	30.5	27
	Post-SDS	0	0.33	1	3	1.5	1
McMillen's	pre-SDS	52	48.5	56	40	41.5	31
	post-SDS	0	0	1	1.5	1.75	1.25
PBS (Control)	pre-SDS	52	48	48	51	50.5	43
	post-SDS	0	0	0	0.25	0.25	0.75
Non-Nutrient Agar	pre-SDS	19.5	22.5	34	38.5	32	25
	post-SDS	0	6	16	35	31	27

Table 8. Comparison of *A. polyphaga* cysts created by various methods surviving 30 minute treatment with 0.25% SDS. 0.5 mL of cells were taken from the various encystment media, centrifuged at 3,000 rcf for 10 minutes, and supernatant was removed. All cells were re-suspended in 500 µL sterile PBS, and then counted both prior to addition of SDS and after 30 minute incubation with SDS. All numbers presented represent averages of three or four different counts**.**

Encystment Method		**Day 0** (# cells per µL)	**Day 1** (# cells per µL)	**Day 2** (# cells per µL)	**Day 3** (# cells per µL)	**Day 4** (# cells per µL)	**Day 5** (# cells per µL)
8% Glucose	pre-SDS	54	41	37	24	14	16.5
	post-SDS	0	0.67	2.75	2.5	2.5	2.25
TRIS	pre-SDS	54	38	28	44.5	43	28
	post-SDS	0	0.33	3.75	7.5	6.5	4.5
Taurine	pre-SDS	54	44	42	36	38.5	42
	Post-SDS	0	0	0.25	1.75	3.75	2.25
McMillen's	pre-SDS	54	44.5	44	45	34.5	30
	post-SDS	0	0.33	7.25	7	4.25	6
PBS (Control)	pre-SDS	54	35	40.5	32	39	28
	post-SDS	0	0.33	1	2.5	3.25	2
Non-Nutrient Agar	pre-SDS	28	30.5	35	28	29	35.5
	post-SDS	0	8.25	18	24	28	37.5

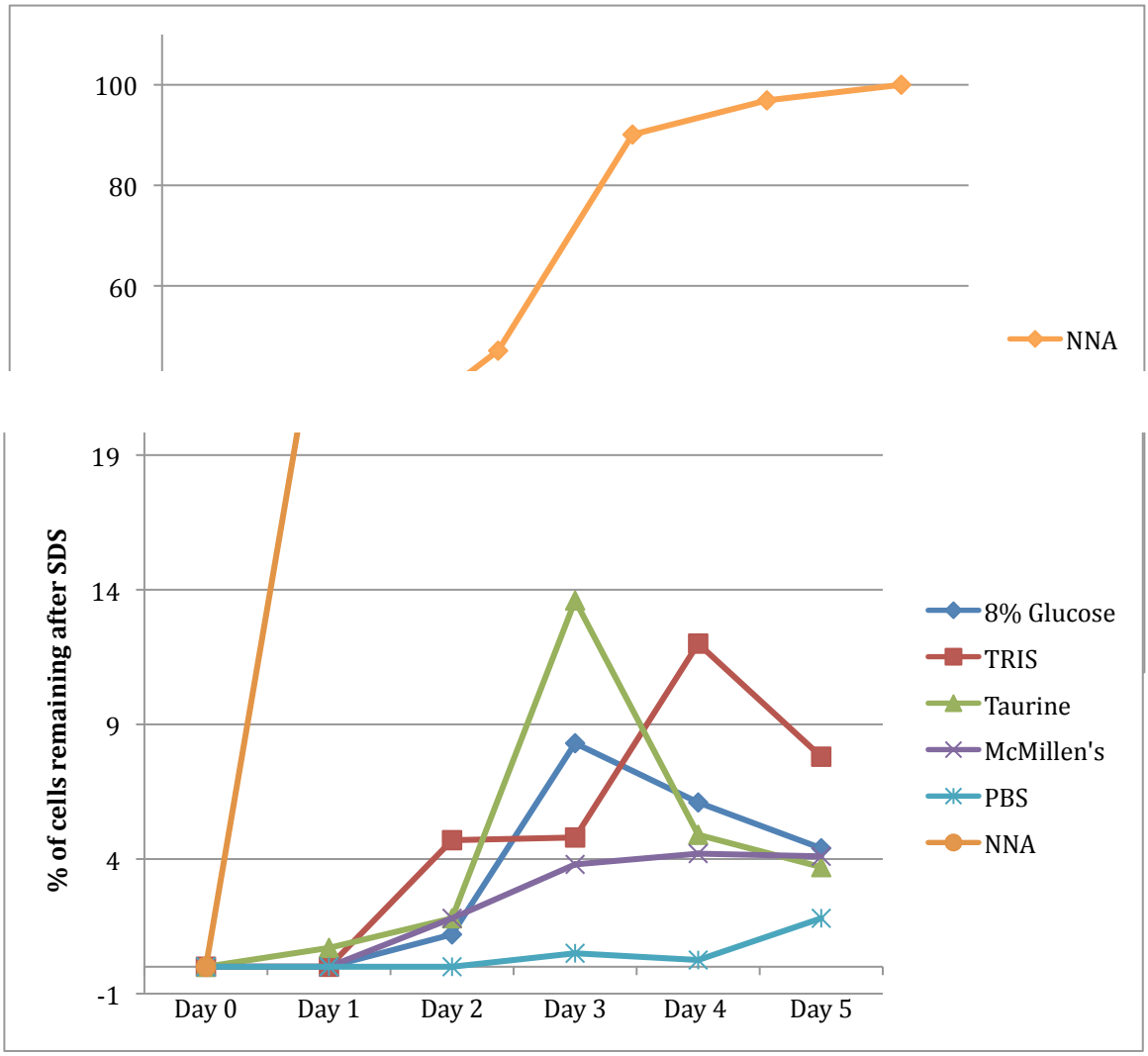

Figure 6. Encystment rate of *A. castellanii* 30234 cells using various encystment media and phosphate buffered saline as a negative control. *A. castellanii* trophs were suspended in encystment media to a final concentration of ~1 x 10^5 cells / mL. Aliquots were removed at the start of the assay (Day 0), and each day after that until 5 days had passed. Each aliquot was centrifuged at 3,000 rcf for 10 minutes, and the supernatant was removed. The cell pellets were then re-suspended in PBS prior to the addition of SDS to a final concentration of 0.25%, and incubated at room temperature for 30 minutes. Cells were counted both before and after SDS addition to determine %age cysts, and each data point represents an average of 4 counts.

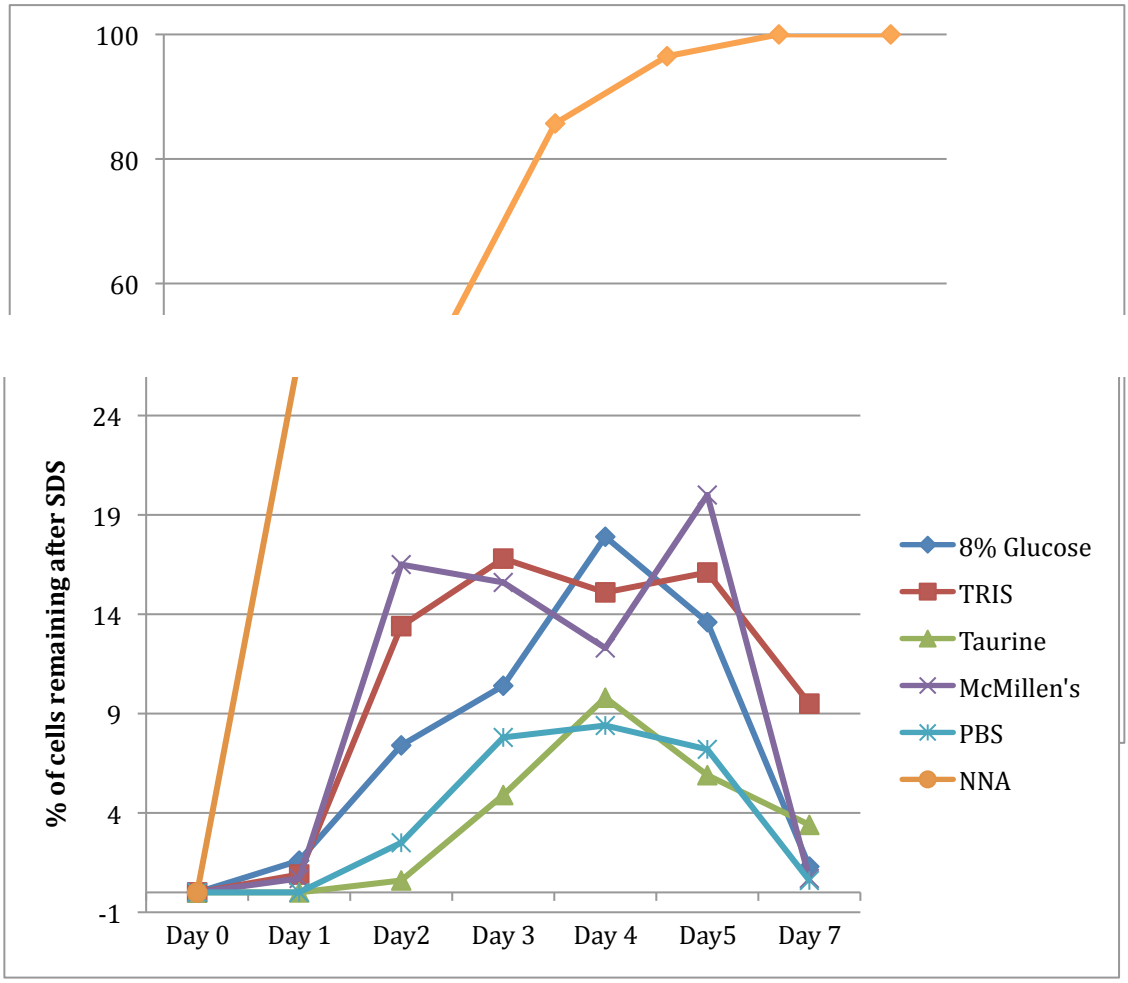

Figure 7. Encystment rate of *A. polyphaga* cells using various encystment media and phosphate buffered saline as a negative control. *A. polyphaga* trophs were suspended in encystment media to a final concentration of ~1 x 10^5 cells / mL. Aliquots were removed at the start of the assay (Day 0), and each day after that until one week had passed. Each aliquot was centrifuged at 3,000 rcf for 10 minutes, and the supernatant was removed. The cell pellets were then re-suspended in PBS prior to the addition of SDS to a final concentration of 0.25%, and incubated at room temperature for 30 minutes. Cells were counted both before and after SDS addition to determine %age cysts, and each data point represents an average of 4 counts.

Results showed encystment in various media peaking between days three and four for *A. castellanii* and *A. polyphaga* (Figures 6 and 7) as measured by counting cells before and after challenge with SDS. Of the encystment methods tested, the non-nutrient agar method had the greatest effect on encysment rates. However, the morphology changes that accompany encystment and allow SDS survival do not indicate the viability of the cells, since some of the cells may no longer be viable. Thus, in order to determine the viability of the cysts that survive SDS treatment, the cyst viability assay was performed.

Viability of cysts created with different encystment methods

Table 9. Results of plaque assay for viability with *A. castellanii* cysts created using different encystment methods.

Encystment Method	**Total *A. castellanii* cysts per mL**	**MPN estimate of viable cells per mL**	**% viability**
PBS	6.0×10^5	1.3×10^5	21.7
Glucose	1.2×10^6	5.4×10^5	45
TRIS	6.5×10^5	1.3×10^5	20.0
Taurine	3.55×10^6	9.2×10^5	25.9
McMillen's	3.3×10^6	3.5×10^5	10.6
NNA	6.5×10^5	4.3×10^5	66.2

Table 10. Results of plaque assay for viability with *A. polyphaga* cysts created using different encystment methods.

Encystment Method	**Total *A. polyphaga* cysts per mL**	**MPN estimate of viable cells per mL**	**% viability**
PBS	2.05×10^6	1.6×10^6	78
Glucose	4.05×10^6	2.4×10^5	5.9
TRIS	3.5×10^6	5.4×10^5	15.4
Taurine	6.35×10^6	9.2×10^5	14.5
McMillen's	2.5×10^6	3.5×10^5	14.0
NNA	3.0×10^6	2.8×10^6	93.3

Results of the viability assay (Tables 9 and 10) showed viability ranging from less than six percent for the hyperosmotic glucose method to nearly 95 percent for the

non-nutrient agar method in *A. polyphaga*. Viability in the *A. castellanii* cysts ranged from just over ten percent using the McMillen's medium to 66.2 percent with the non-nutrient agar method of encystment. As with the rates of encystment, the highest viability levels were seen with the use of the non-nutrient agar method of encystment for both species. Chemical methods of encystment resulted in two to four times fewer viable cysts. The subsequent experiments examining gene expression of encystment genes were carried out in order to compare events occurring during the encystment processes. The TRIS chemical method was chosen for comparative purposes due to the fact that it produced the highest viability levels in *A. polyphaga*, as this was the species with the fewest viable chemically-induced cysts (Figure 8).

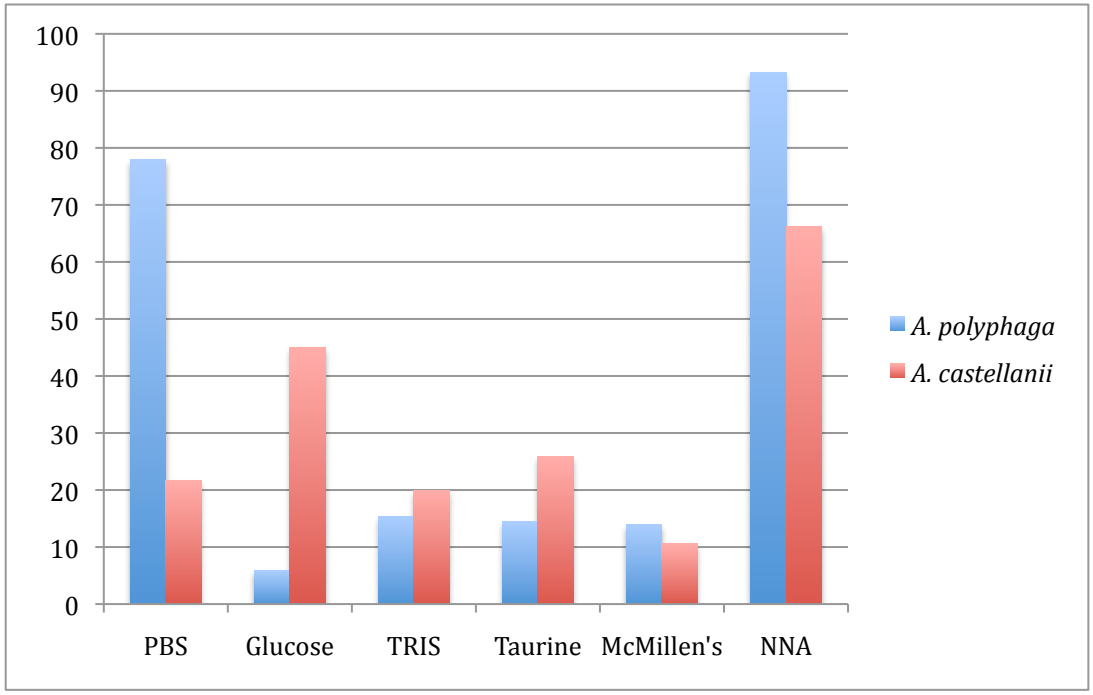

Figure 8. Percentage of cysts created by different methods of encystment that are viable as estimated using the plaque assay method and the FDA's MPN table. For each method, total cell numbers were counted after 5 day incubations in the encystment medium. Serial dilutions of the cysts were then created, and 10 μL spots were plated onto plaque assay plates. Numbers of clearings were counted and used to estimate number of viable cells per spot; this number was then used in the following calculation to determine viability: (# of viable cells per mL / # of total cells per mL) x 100.

PCR screening of primer library

In order to determine which primer sets would be successful in the gene expression RT-PCR reactions, PCR was performed with all primer sets using genomic DNA extracted from each species. In this way, ideal primers for RT-PCR could be selected.

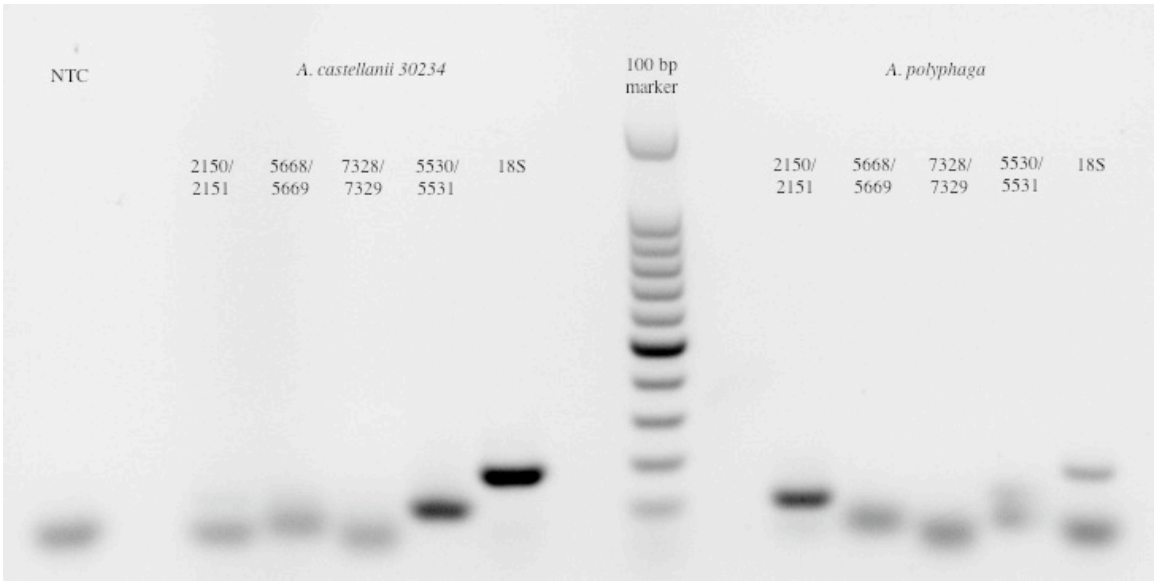

Figure 9. AGE of primer library screening of primers targeting serine protease. For each reaction, 50 ng of template DNA and 1 μL of 10 μM primer was used. The thermal cycle program was 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 90V for 100 minutes.

The primer set 5530/5531 successfully amplified a region of the serine protease gene in *A. castellanii*; similarly, the primer set 2150/2151 amplified a region of the serine protease gene in *A. polyphaga*. These primer sets were therefore chosen for the RT-PCR reactions to examine expression of this gene.

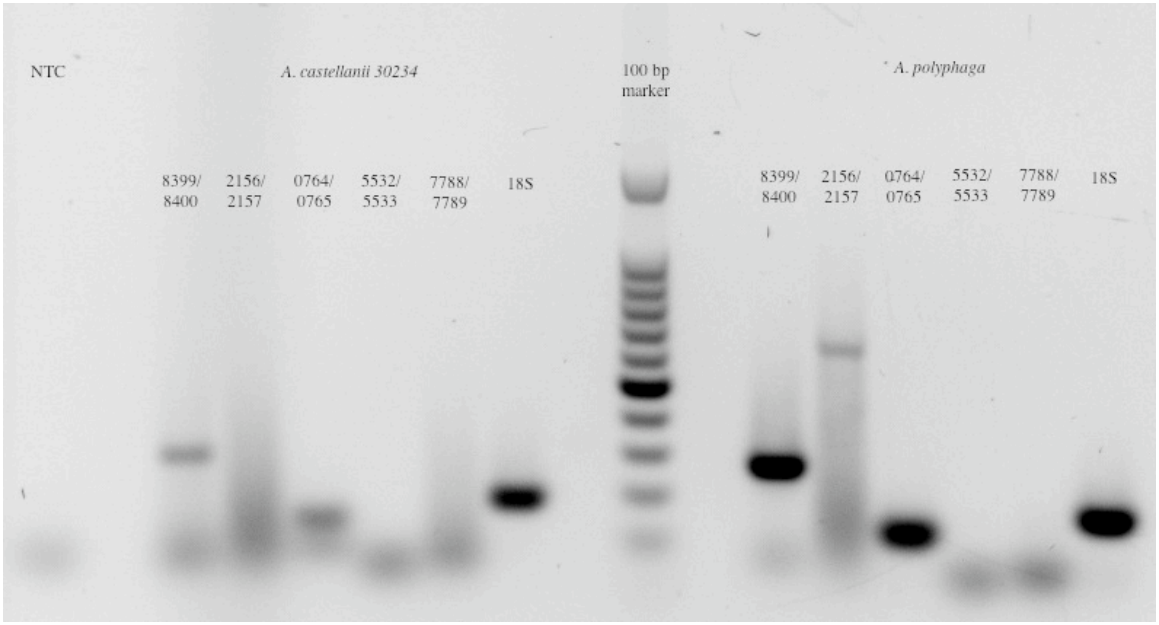

Figure 10. AGE of primer library screening of primers targeting glycogen phosphorylase. For each reaction, 50 ng of template DNA and 1 μL of 10 μM primer was used. The thermal cycle program was 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72C for 1 minute and 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 90V for 100 minutes.

The primer set 8399/8400 successfully amplified a region of the glycogen phosphorylase gene in *A. castellanii*; the same primer set amplified a region of the glycogen phosphorylase gene in *A. polyphaga*. Therefore, these two primer sets were selected for the RT-PCR reactions to examine expression of this gene.

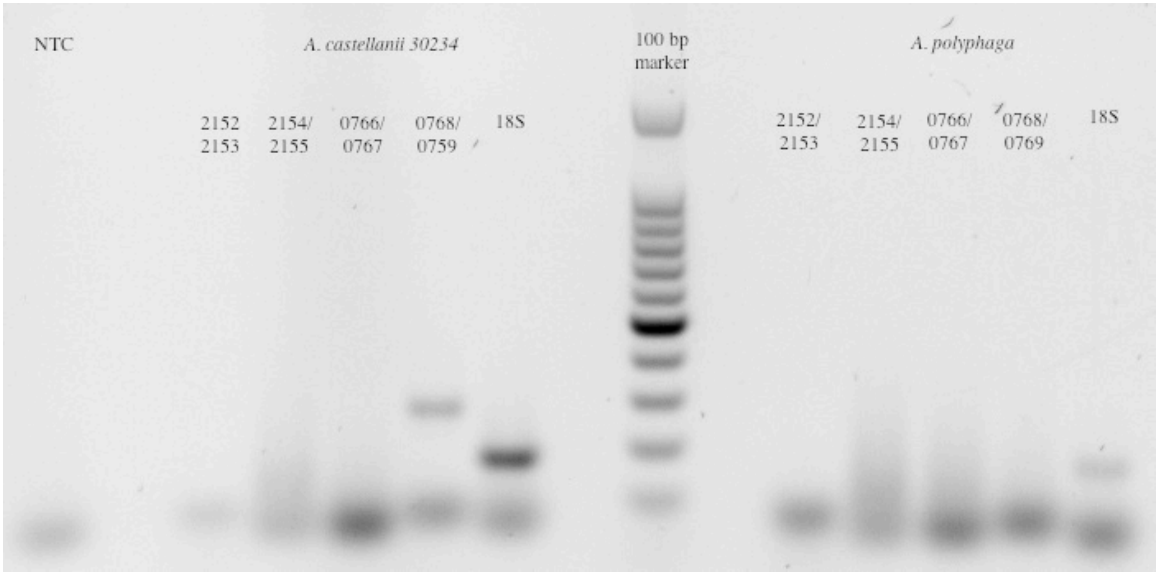

Figure 11. AGE of primer library screening of primers targeting cellulose synthase. For each reaction, 50 ng of template DNA and 1 μL of 10 μM primer was used. The thermal cycle program was 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 90V for 100 minutes.

The primer set 0768/0759 successfully amplified a region of the cellulose synthase gene in *A. castellanii*; none of the screened primer sets amplified a region of the cellulose synthase gene in *A. polyphaga*. Therefore, this primer set was selected for the RT-PCR reactions to examine expression of this gene in *A. castellanii*.

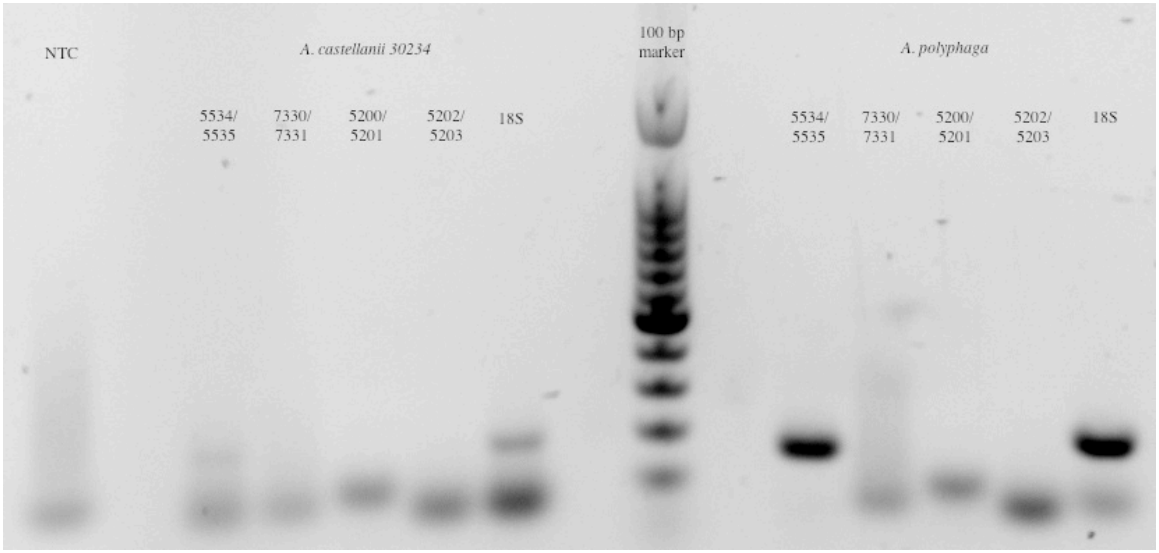

Figure 12. AGE of primer library screening of primers targeting enolase. For each reaction, 50 ng of template DNA and 1 μL of 10 μM primer was used. The thermal cycle program was 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 90V for 100 minutes.

The primer set 5534/5535 successfully amplified a region of the cellulose synthase gene in *A. polyphaga*; none of the screened primer sets amplified a region of the cellulose synthase gene in *A. castellanii*. Therefore, this primer set was selected for the RT-PCR reactions to examine expression of this gene in *A. polyphaga*.

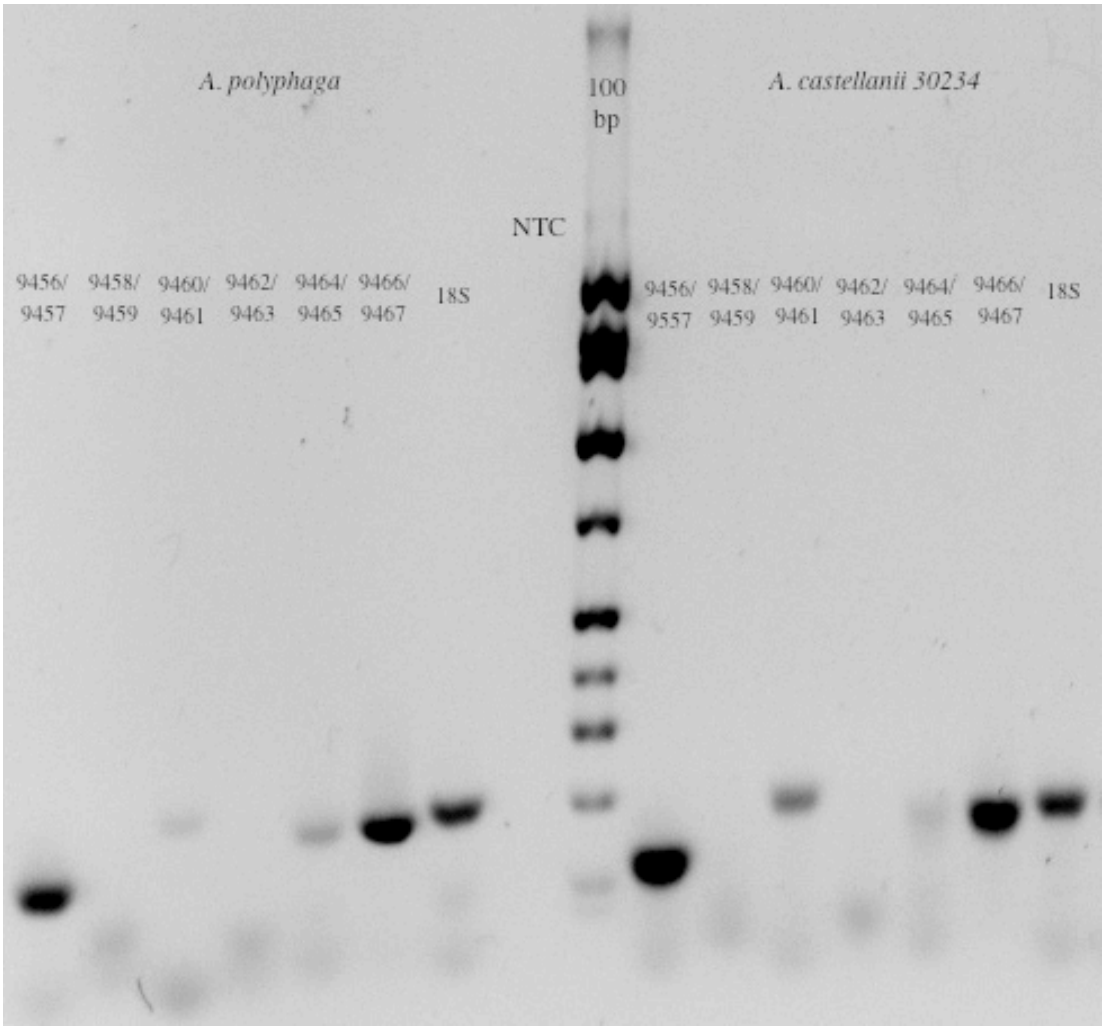

Figure 13. AGE of primer library screening of primers targeting potential reference genes. For each reaction, 50 ng of template DNA and 1 μL of 10 μM primer was used. The thermal cycle program was 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 90V for 100 minutes.

The primer sets 9456/9457, 9460/9461, 9464/9465, and 9466/9467 all successfully amplified a region of a potential reference gene in *A. castellanii* and *A. polyphaga*. Therefore, these primer sets were selected for the RT-PCR reactions to examine expression of these genes in both species.

RT-PCR

RT-PCR reactions were carried out in order to examine gene expression in both *Acanthamoeba* species during different methods of encystment. RNA extractions were taken at various time points during encystment and used as template for the reactions with the primer sets determined by the PCR reactions described previously. In this way, gene expression could be compared during encystment processes. Due to vastly differing viabilities of the cysts produced using the different methods, gene expression was expected to vary during this assay; indeed, variations in the duration of expression of the target genes involved in encystment were observed in *A. castellanii* (Figures 14 and 17) and *A. polyphaga* (Figures 15 and 18).

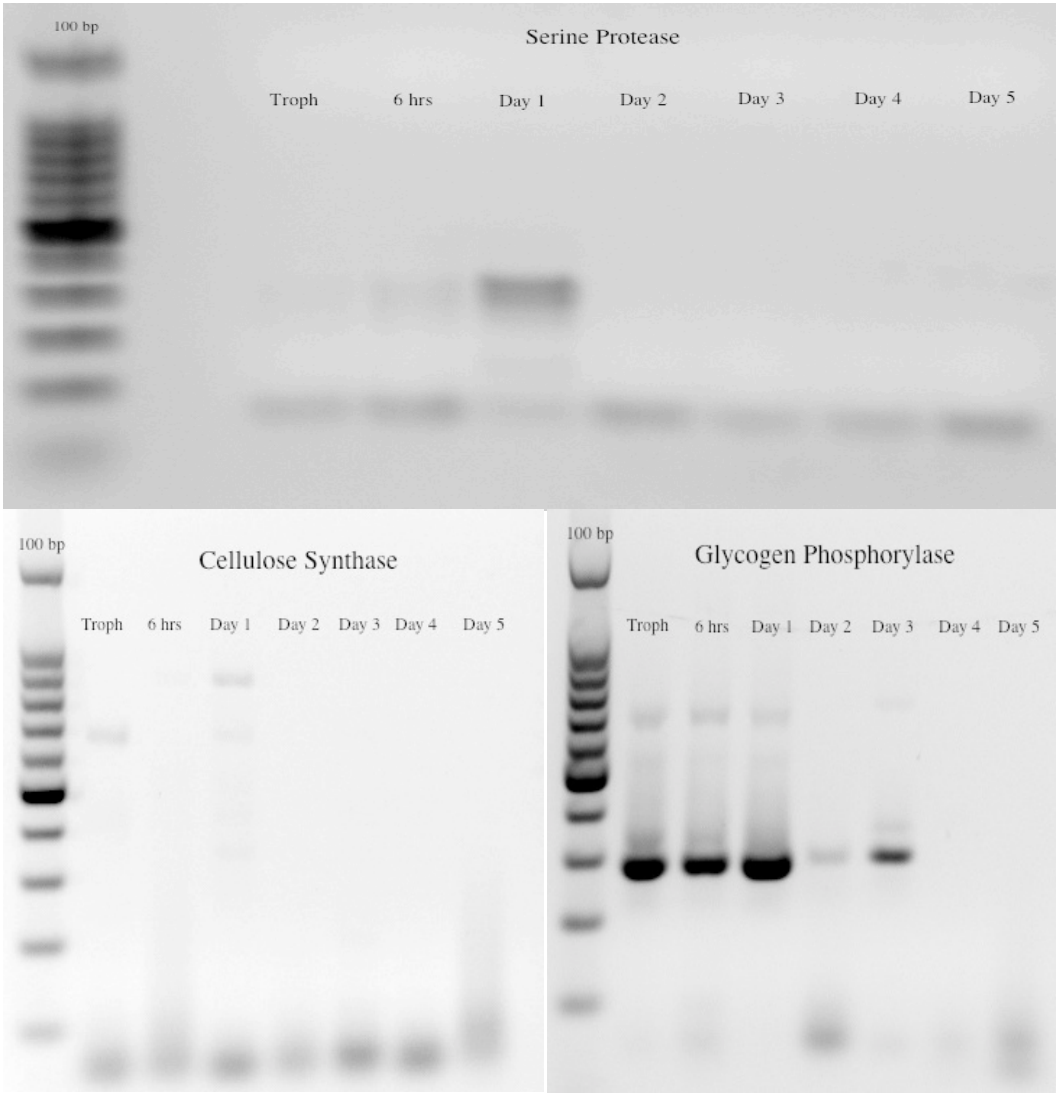

Figure 14. AGE of RT-PCR reactions targeting encystment genes in *A. castellanii* during encystment induced using the NNA method. For each reaction, 10 ng of template RNA and 0.5 μL of 10 μM primer were used. The thermal cycle program was 68°C for 30 minutes, 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of 1.2% agarose gels and run at 90V for 100 minutes.

Banding patterns indicating the presence of the glycogen phosphorylase gene were visible in the trophozoite and encystment RNA extraction up to day three in *A. castellanii*. No bands were visible after this point. Similarly, amplicons were observed in the six hour and day one RNA samples from this organism for the serine protease gene. The primers used for the RT-PCR reactions to target the cellulose synthase gene did not successfully amplify a target of the expected size; instead, multiple, non-specific bands were observed in the troph and day one lanes.

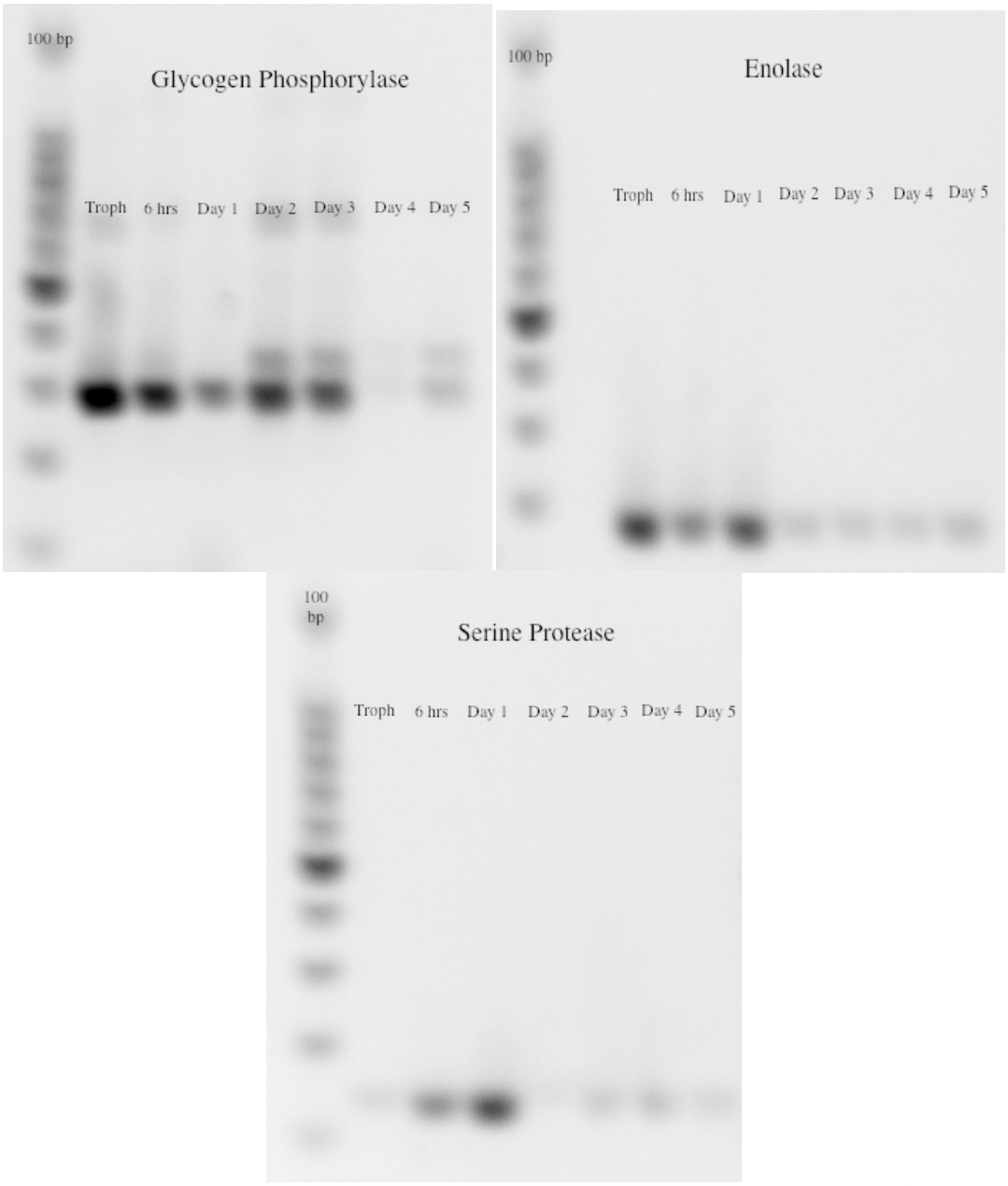

Figure 15. AGE of RT-PCR reactions targeting encystment genes in *A. polyphaga* during encystment induced using the NNA method. For each reaction, 10 ng of template RNA and 0.5 µL of 10 µM primer were used. The thermal cycle program was 68°C for 30 minutes, 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of 1.2% agarose gels and run at 90V for 100 minutes.

Banding patterns indicating the presence of glycogen phosphorylase gene were observed in the trophozoite and encystment (using the NNA method) RNA extraction up to day three in *A. polyphaga*. Only light bands were visible after this point. Similarly, amplicons were observed in the six hour and day one RNA samples from this organism for the serine protease gene. The reactions targeting the enolase gene in *A. polyphaga* showed heavier banding in the trophozoite, six hour, and day one RNA samples and some lighter banding in the remaining samples.

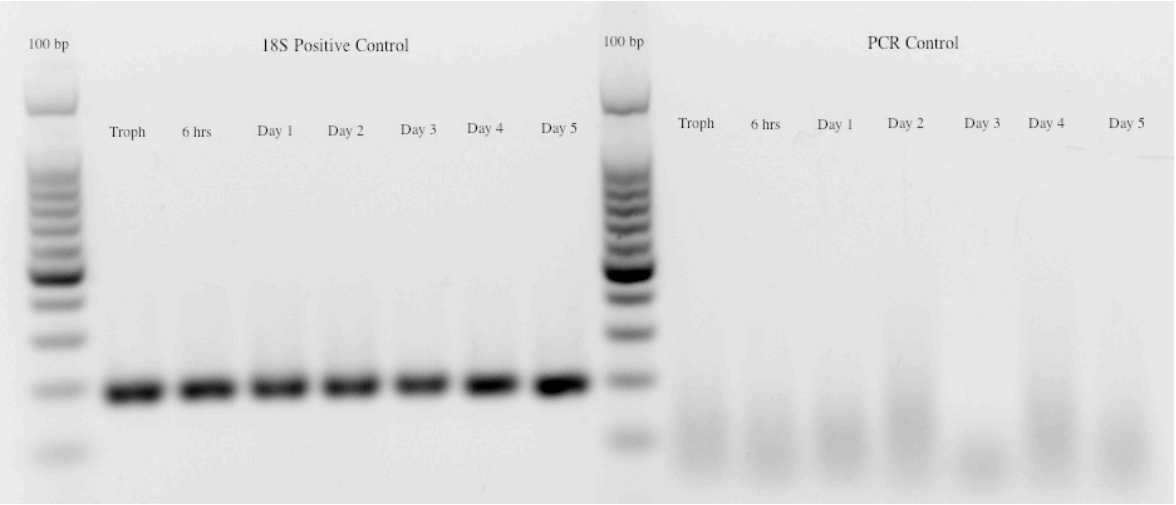

Figure 16. AGE of RT-PCR control reactions for *A. castellanii* during encystment induced using the NNA method. For each reaction, 10 ng of template RNA and 0.5 μL of 10 μM primer were used. The thermal cycle program was 68°C for 30 minutes, 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of 1.2% agarose gels and run at 90V for 100 minutes.

The control 18S RT-PCR reaction showed positive banding in all samples of RNA (Figures 16 and 19); the PCR reaction using the same primer set and no reverse transcriptase resulted in no amplification in any of the samples.

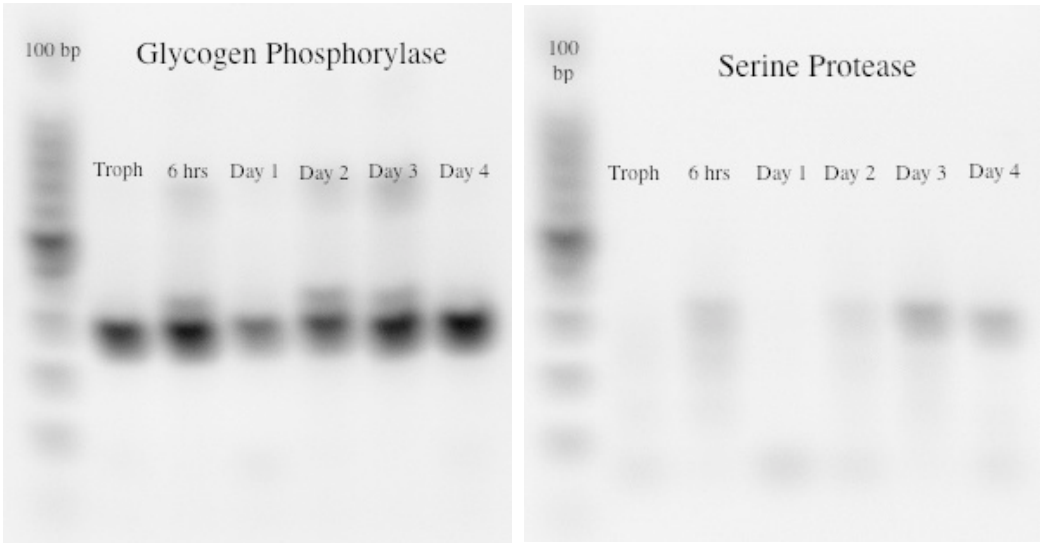

Figure 17. AGE of RT-PCR reactions targeting encystment genes in *A. castellanii* during encystment induced using the TRIS chemical induction method. For each reaction, 10 ng of template RNA and 0.5 μL of 10 μM primer were used. The thermal cycle program was 68°C for 30 minutes, 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of 1.2% agarose gels and run at 90V for 100 minutes.

Banding patterns indicative of the glycogen phosphorylase gene were visible in the trophozoite and encystment (using the TRIS method) RNA extraction for all samples in *A. polyphaga*. Amplicons indicating the presence of mRNA's for serine protease were observed in all of the samples with the exception of the sample from day one.

Figure 18. AGE of RT-PCR reactions targeting encystment genes in *A. polyphaga* during encystment induced using the TRIS chemical induction method. For each reaction, 10 ng of template RNA and 0.5 µL of 10 µM primer were used. The thermal cycle program was 68°C for 30 minutes, 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of 1.2% agarose gels and run at 90V for 100 minutes.

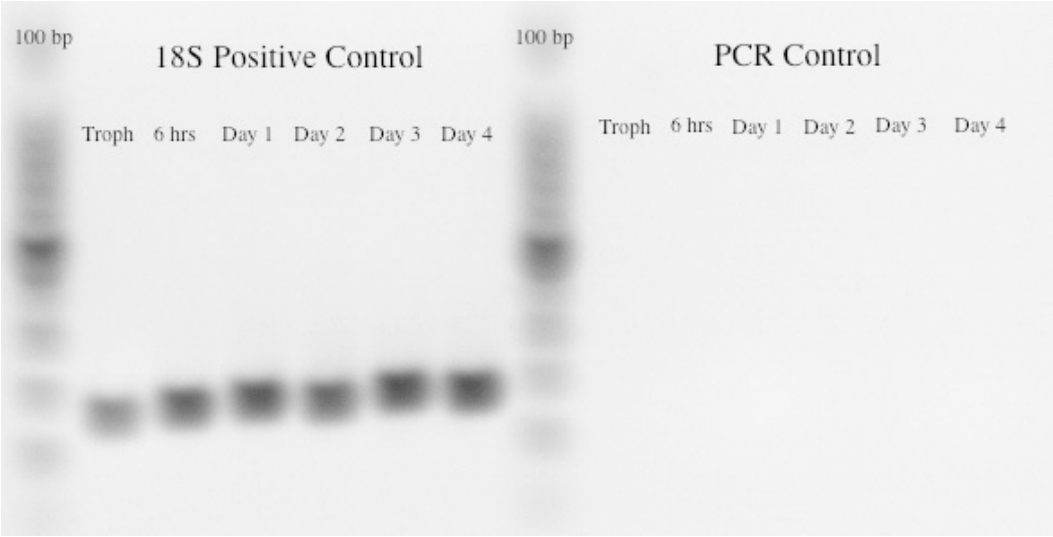

Figure 19. AGE of RT-PCR control reactions for *A. castellanii* during encystment induced using the TRIS chemical induction method. For each reaction, 10 ng of template RNA and 0.5 μL of 10 μM primer were used. The thermal cycle program was 68°C for 30 minutes, 94°C for 5 minutes, 40 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of 1.2% agarose gels and run at 90V for 100 minutes.

Randomly Amplified Polymorphic DNA (RAPD) Typing

Acanthamoeba typing methods using various techniques, such as RFLP or 18S DNA sequencing, take days to distinguish isolates, at best. Therefore, the RAPD method was optimized in the following reactions for the purposes of providing a faster means of identification of *Acanthamoeba* isolates.

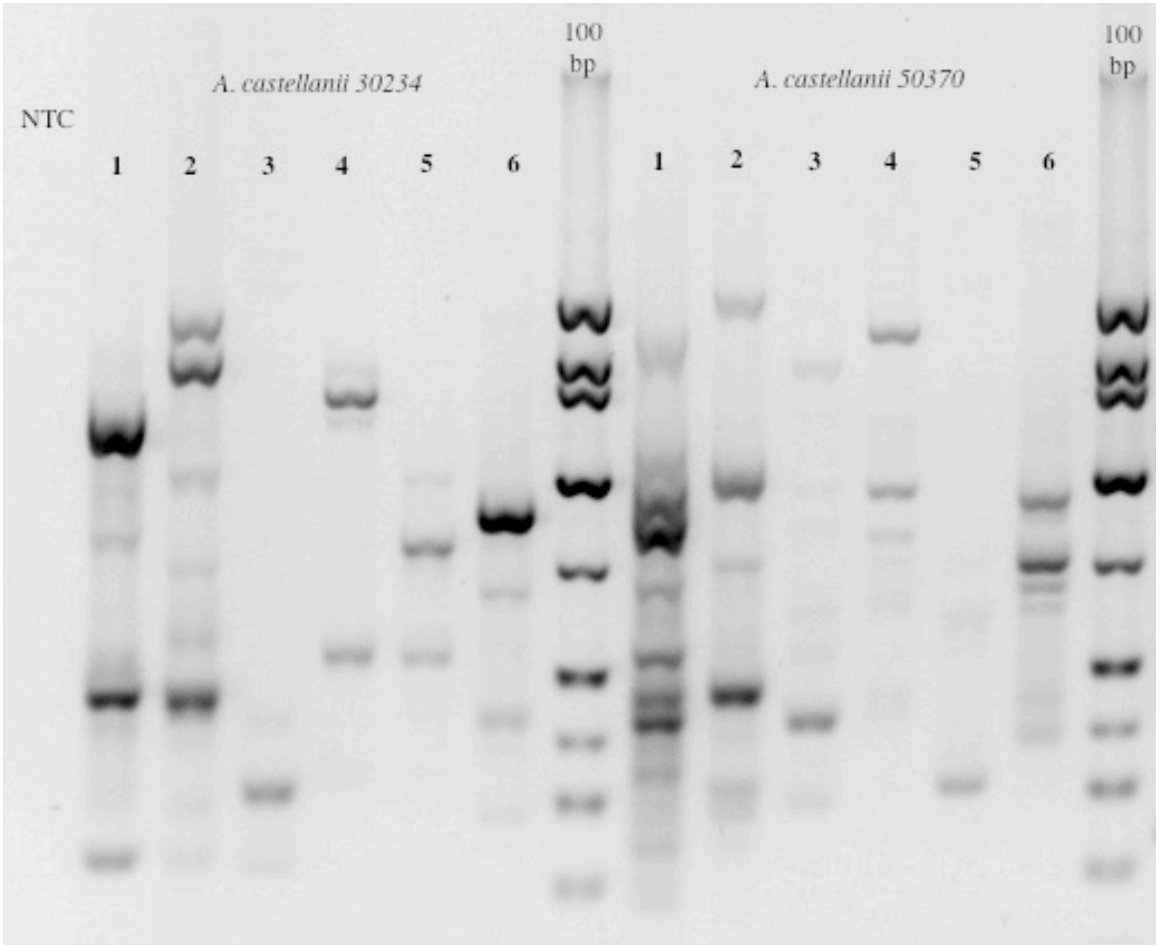

Figure 20. AGE of RAPD-PCR comparing *A. castellanii* ATCC 30234 with *A. castellanii* 50370. For each strain, ~50 ng of template DNA was used in a PCR reaction with six different 10mer primers, described in Table 6. The thermal cycle program was 94°C for 5 minutes, 45 cycles of (94°C for 30 seconds, 35°C for 30 seconds, 72°C for 1 minute and 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 90V for 100 minutes.

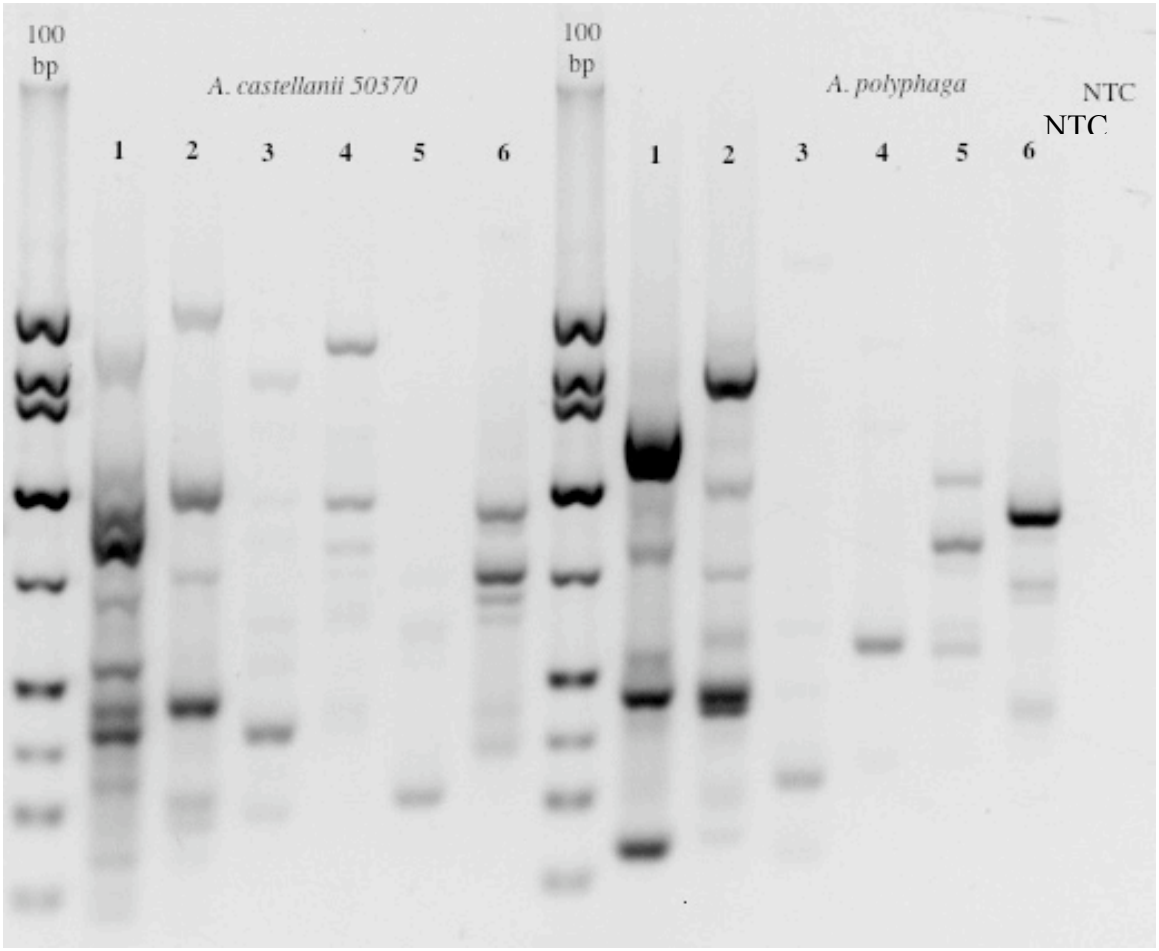

Figure 21. AGE of RAPD-PCR comparing *A. castellanii* ATCC 50370 with *A. polyphaga*. For each strain, 50 ng of template DNA was used in a PCR reaction with six different 10mer primers, described in Table 6. The thermal cycle program was 94°C for 5 minutes, 45 cycles of (94°C for 30 seconds, 35°C for 30 seconds, 72°C for 1 minute and 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 90V for 100 minutes.

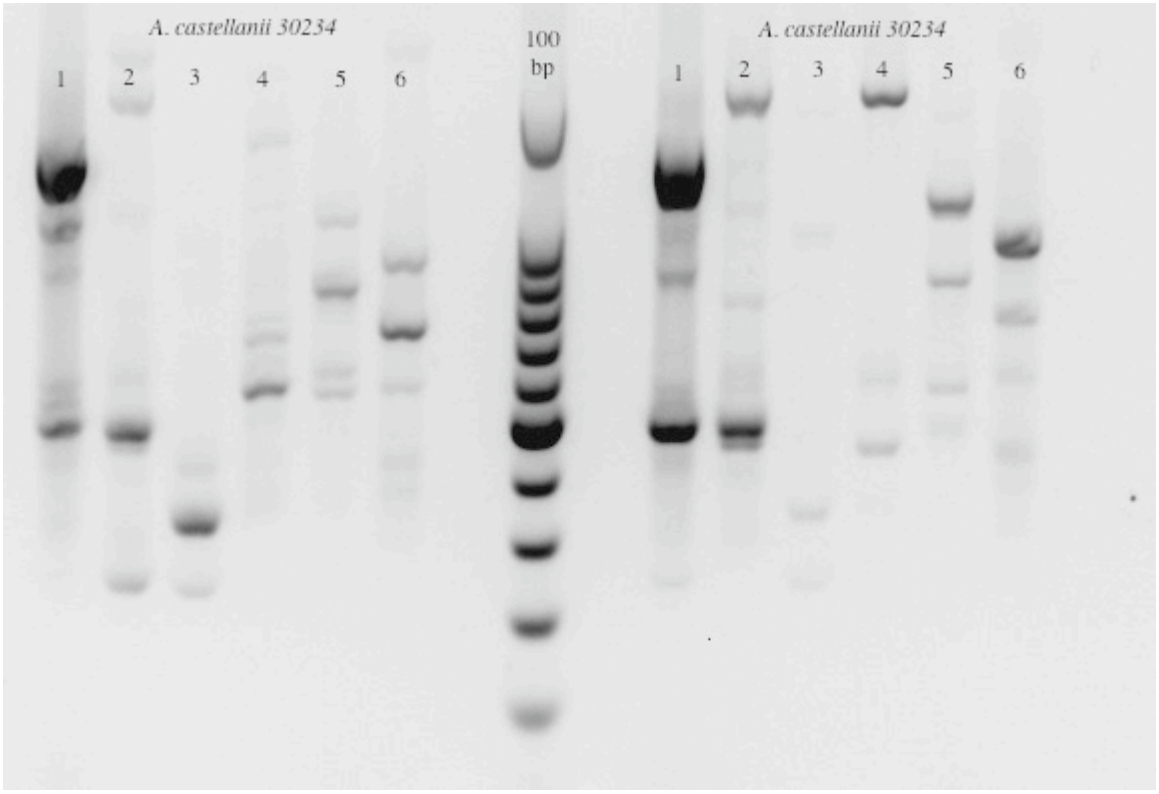

Figure 22. AGE of RAPD-PCR comparing two different preparations of DNA from A. castellanii 30234. For each preparation, 25 ng of template DNA was used in a PCR reaction with six different 10mer primers, described in Table 6. The thermal cycle program was 94°C for 5 minutes, 45 cycles of (94°C for 30 seconds, 35°C for 30 seconds, 72°C for 1 minute 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 90V for 100 minutes.

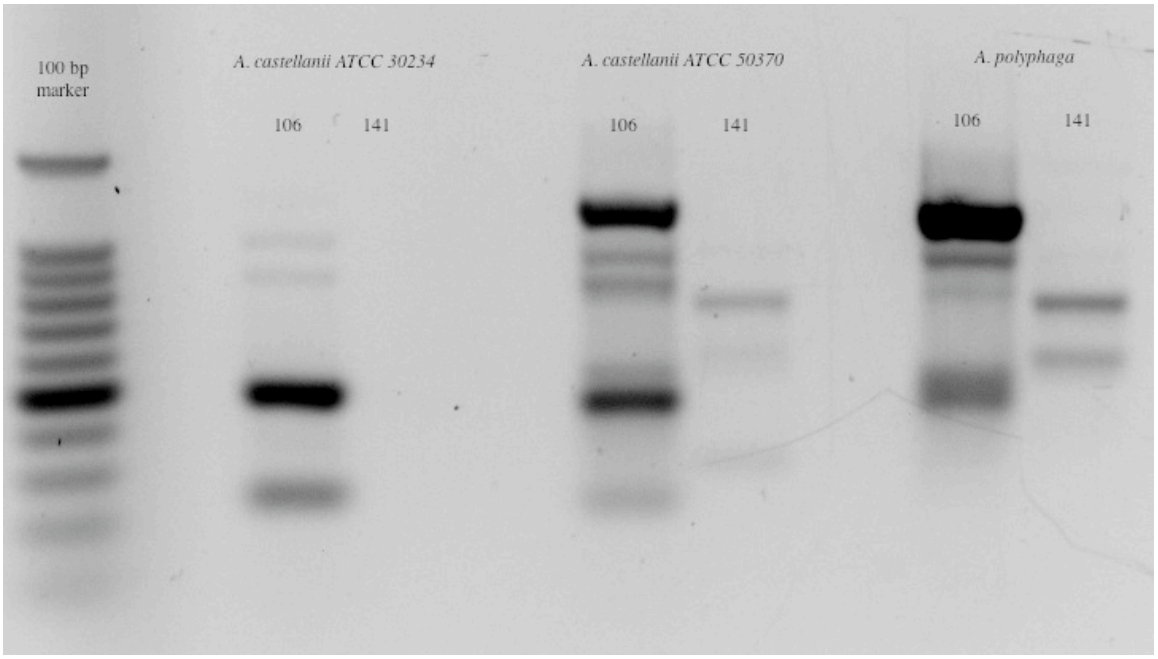

Figure 23. AGE of RAPD-PCR using the two primers selected for their consistency (106 and 141) and 25 ng of template DNA from *A. castellanii* 30234, *A. castellanii* 50370, and *A. polyphaga*. The thermal cycle program was 94°C for 5 minutes, 45 cycles of (94°C for 30 seconds, 35°C for 30 seconds, 72°C for 1 minute and 30 seconds), 72°C for 10 minutes. PCR product was then loaded into wells of a 1.2% agarose gel and run at 120V for 70 minutes.

Multiple amplicons were observed using the primers for the RAPD reactions. Patterns could be compared for identification purposes between species (Figure 21) and between strains of the same species (Figure 20). Further comparison of the banding patterns produced with different DNA extractions of the same species and strain were made in order to select primers that exhibited consistent amplification (Figure 22); using these primers, a final set of PCR reactions was run (Figure 23). This final set proved to provide a consistent pattern of amplicons that could be used for typing isolates of *Acanthamoeba*.

DISCUSSION

Comparison of encystment methods

While the chemical encystment media showed surprisingly low levels of inducing encystment, with none producing more than 20% cysts during the 5-day incubation, the non-nutrient agar method was able to consistently produce 100% encystment in both species tested. It has previously been reported that *Acanthamoeba* that are axenically cultivated over long periods of time lose their ability to encyst synchronously (Kohsler et al. 2008), and this does seem to be confirmed in this study with the use of chemical encystment media. Thus, when cyst induction is required by investigation, it would be prudent to use the non-nutrient agar method rather than one of the many chemical induction methods.

A comparison of *A. castellanii* 30234 and *A. polyphaga* encystment revealed that the *A. polyphaga* cells had higher encystment rates in all of the media, as determined by the treatment of cells during the course of encystment with 0.25% SDS. While other published studies have used 0.5% SDS to lyse trophozoite cells (Cordingly et al. 1996; Dudley et al. 2007), experiments with these particular strains revealed 0.25% SDS to be adequate, and less likely to interfere with downstream viability assays.

Figures 6 and 7 show graphical representations of encystment rates of *A. castellanii* and *A. polyphaga*. For both strains and all encystment media, peak encystment occurred somewhere between days 3 and 4 of the assay. This should be considered in future studies for optimum harvesting of cysts. The decrease in the percentage of cysts after that peak could be due to the triggering of apoptosis by the stress

conditions created by the media. Gao and Kwaik (2000) demonstrated that *Acanthamoeba* do undergo apoptosis in response to certain conditions, and it is thus a possible explanation for the loss of cells from the media.

Relatively low encystment rates were observed in the control PBS solution; starvation conditions in the PBS cell suspension might trigger encystment in some of the cells. Also, decreasing overall numbers of cells, as shown in Tables 6 and 7, might be due to their cannibalistic tendencies, as food is no longer provided in the PBS. Another possible explanation would be the induction of the apoptotic pathway. As some cells may disappear due to lysis, the release of intracellular components might act as a trigger for apoptosis. While lysis would be expected to occur relatively quickly once cells are exposed to the encystment media, a decrease in cell number was observed for several days. Thus, apoptosis is likely involved. Comparison of the rates between *A. polyphaga* and *A. castellanii* in PBS again reveals the higher capacity of the *A. polyphaga* cells to encyst, perhaps as they might have lost less of their encystment capabilities due to prolonged axenic culture and the removal of selective pressures for these capabilities.

Viability of cysts created with different encystment methods

Tables 9 and 10 show the numbers and percentages of viable cysts created in *A. castellanii* and *A. polyphaga* using various encystment media, as well as the non-nutrient agar drying method of producing cysts. Clearly, the non-nutrient agar technique produced a significantly greater percentage of viable cysts as compared to the chemical encystment media. Using the NNA method with *A. polyphaga,* the population of cysts

approaches total viability (93.3%), while about two thirds of the *A. castellanii* population is viable.

Analysis of the capacity of the encystment media to create viable cysts reveals that the hyperosmotic glucose solution produces the highest percentage of viable cysts in *A. castellanii*, but the lowest percentage of viable cysts in *A. polyphaga*. Also, the alkaline TRIS encystment media produced the highest percentage of viable cysts in *A. polyphaga*. These findings suggest that the use of a single encystment media for all species of *Acanthamoeba* in order to produce cysts for downstream testing is misguided; the ideal cyst production method clearly varies from species to species.

The data as shown in Figure 8 reveal the variation in the methods of encystment media in their ability to produce viable cysts. Interestingly, while the encystment media produce larger numbers of cysts in *A. polyphaga* as compared to *A. castellanii* (Figures 6 and 7), a larger percentage of those cysts are viable in *A. castellanii*, with the exception of those cysts produced using McMillen's medium. The numbers of cysts that exhibited viability using this assay were surprisingly low for the chemical encystment media, with an average of less than 15% of *A. polyphaga* cysts excysting and an average of less than 30% of *A. castellanii* cysts excysting.

It is clear that the NNA method of encystment is the ideal choice for producing cysts, as 100% encystment occurs between days three and four for both species tested (Tables 7 and 8), and these cysts are two to five times more likely than any produced using chemical induction to be viable. Thus, while there is currently no extant protocol approved by the FDA for production of cysts to be used in contact lens solution testing,

these data present a strong case for the adoption of the non-nutrient agar method of cyst production.

PCR screening of primer library

Screening of the primer library with PCR revealed primer sets that would work for four different encystment genes, as well as three potential reference genes for future RT-PCR work. A portion of the serine protease was successfully amplified with the primer set 5530/5531 in *A. castellanii*, and the primer set 2150/2151 in *A. polyphaga*. The primer set 0764/0765 worked for both *A. castellanii* and *A. polyphaga* to amplify a region of the glycogen phosphorylase gene. The cellulose synthase gene was targeted successfully only in *A. castellanii*, by the primer set 0768/0759. Similarly, the enolase gene was targeted successfully only in *A. polyphaga*, by the primer set 5534/5535.

As only one of the primer sets amplified a target region in both organisms, the genetic differences between the two are significant to expression studies. As such, any future studies of this nature in *Acanthamoeba* are unlikely to be successful across multiple species and strains. For this reason, investigations that elucidate sequences and expression patterns for these organisms will be of importance to the understanding of genetic regulation involved in the encystment process.

RT-PCR

In order to quantify real time PCR data, a reference gene must be selected for comparative purposes and normalization. While previous expression studies in *Acanthamoeba* have utilized 18S ribosomal DNA targets for the reference gene (Moon et al. 2008b), levels of expression are relatively high and could result in an inability to

compare genes expressed at a low level for normalization purposes. For that reason, other candidate reference genes were chosen based on their likelihood to be expressed in both the trophozoite and cyst forms of *Acanthamoeba*. Targets in metabolic and structural genes (Table 4) were screened using PCR and then RT-PCR to get an idea of relative expression levels. PCR screening revealed that the primer sets targeting myosin, cytochrome oxidase subunit 2, and profilin were most likely to produce amplicons useful for normalization purposes (Figure 11).

The RNA extracted during the course of encystment for each of the species was analyzed using RT-PCR, and thus comparisons could be made regarding gene expression of target encystment genes. Figures 16 and 19 show positive and negative controls included for each RNA sample; positive controls using the primer set 4772/4773 which targeted a portion of the 18S ribosomal RNA clearly showed banding in all of the lanes for all of the organisms tested, indicating the quality of all RNA samples tested. Similarly, PCR controls included for each sample using the same primer set but with no reverse transcriptase enzyme clearly show no banding for any sample; this indicates the lack of DNA contamination of the samples tested.

Figure 14 gives a picture of gene expression of glycogen phosphorylase and serine protease in *A. castellanii* during encystment induced by drying, or the NNA method. While primer screening of DNA of *A. castellanii* revealed a primer set for targeting the ellulose synthase gene, the RT-PCR reaction using this primer set did not produce amplicons consistent with the expected size; the minor banding which can be observed in the first few lanes of the gel can be explained by minor non-specific

annealing that might occur as a result of RNA processing at the post-transcriptional level. For this reason, the reverse primer that was used to target the DNA might not successfully bind to the complementary RNA if it landed in an intron region. As this gene has not yet been sequenced and characterized, further investigation is required to present a pattern of cellulose synthase expression. As this gene would be absolutely necessary for the synthesis of the cellulosic inner wall of the cyst, its activity in the encysting cell is indisputable, and further work should be done to determine its expression pattern. Furthermore, its presence has been indicated by shotgun sequencing of the genome (Anderson et al. 2005). It could be predicted, however, that expression should be active concurrently and subsequently to the activity of glycogen phosphorylase, since the liberation of glucose is necessary for the building of cellulose.

Interestingly, the glycogen phosphorylase gene expression was evident in the troph forms of both *A. castellanii* and *A. polyphaga* (Figures 14, 15, 17, and 18). While glucose must be liberated for the formation of the cell wall when encystment is induced, it appears to also be necessary in the troph for general glucose cycling during normal metabolism. Building of the cellulosic wall happens early during encystment; thus it is reasonable to expect expression during early encystment (Khan 2006). Generally, glycogen phosphorylase was also shown to be active upon induction of encystment, and through the first few days of the process. By the time 100% of the population had encysted in *A. castellanii*, glycogen phosphorylase expression was no longer apparent. Similarly, expression of glycogen phosphorylase was not strong past day three for *A. polyphaga*. Interestingly, this pattern of expression changed with the use of the TRIS

chemical encystment media. Both species in this media exhibited expression of glycogen phosphorylase for all samples taken during this process (Figures 17 and 18). While trophs were lysed with SDS prior to RNA extraction, it is possible that early cysts were present over the entire course of extraction. If a significant portion of the cell population started encysting somewhat later during the chemical induction, this expression could be explained by the presence of those cells in the later days of the induction and extraction. Examination of glycogen phosphorylase gene expression reveals that the encystment process is most likely to be synchronous in the population when induced with the non-nutrient agar method of encystment, as compared to the chemical method of encystment.

This pattern holds true with the examination of the serine protease gene as well. For both *A. castellanii* and *A. polyphaga*, expression of the serine protease is highest on Day 1 of the NNA method of encystment, with some increased expression by six hours after induction (Figures 14 and 15); expression drops off after this point for each of these species. However, during the TRIS induction of encystment, expression of this gene can be seen throughout the process, similar to that of glycogen phosphorylase (Figures 17 and 18). A similar pattern can be determined for expression of the enolase gene in *A. polyphaga*, wherein higher expression is apparent in the first few days using the NNA method, while more even expression over the course of the experiment is visible for the TRIS method of induction. Indeed, the method of encystment had a greater impact on variability of gene expression than did difference in species; both species exhibited similar patterns with the different encystment methods.

Based on the rates of encystment, viability, and gene expression during encystment, it would seem reasonable to recommend the use of the non-nutrient agar method for the production of cysts for study. While there is no established protocol for making cysts for purposes such as challenge with contact lens cleaning solutions, clearly there is great variability in the quality of those cysts dependent on the mechanism by which they are produced.

RAPD Typing

The 10-mer primers used in this study produced different banding patterns between species (Figure 21) and strains of the same species (Figure 20) based on random amplification of genomic regions complementary to these sequences. Presumably, identification at the strain level could be done for unknown isolates using these primers in a relatively quick (less than 5 hours from start to finish) manner. Indeed, this technique has been used to type a wide range of organisms, including fungi, plants, and arthropods (Aufauvre-Brown et al. 1992; Arif et al. 2010; Guerra et al. 2010). In order to address the concern raised by others over the consistency of this method for typing organisms, a RAPD-PCR reaction using these primers was run with two different preparations of DNA from the same organism (*A. castellanii* 30234), using cells taken from the same flask. Thus, any variation in banding pattern from this gel could be attributed to inconsistency in primer annealing. The gel (Figure 22) did reveal different banding patterns in several of the primers, and two were chosen for their relative consistency (primers 106 and 141). Thus, the RAPD-PCR technique was refined to use only the two primers that produced consistent banding patterns; these two primers were still sufficient to reveal differences

between both strains (*A. castellanii* 30234 and *A. castellanii* 50370) and species (*A. polyphaga* and *A. castellanii*), as shown in Figure 23. There is potential diagnostic application of this technique in that different strains or species of *Acanthamoeba* could be quickly identified, specifically and consistently using these two primers. This approach to typing offers the obvious advantage of speed over other techniques such as RFLP or 18S ribosomal DNA sequencing, both of which take days to complete.

2 EVALUATING THE BIO-BUS PROGRAM: IMPACT ON STUDENT CONFIDENCE AND INTEREST IN SCIENCE

2.1 INTRODUCTION

Science Education in Georgia

The current state of public education in Georgia could be described as dismal at best. While the high school graduation rate has been slightly increasing in the last decade, still more than 20% of students do not graduate (Georgia Department of Education 2009). While this information is disheartening, the statistics relevant to the subject area of science are even more abysmal. State-reported data indicate that a significant number of our students are simply not learning science. For instance, in 2009, 36% of all students failed to demonstrate proficiency in Biology on the End-of-Course Test (EOCT) mandated for all high school students. Similarly, 30% of all students failed to demonstrate proficiency on the Physical Science EOCT (Georgia Department of Education). For the 2008-2009 school year, 36% of 8th grade students did not meet the minimum proficiency level standards in science for the Criterion-Referenced Competency Test (CRCT); in the 2007-2008 school year, 40% of the students failed the science portion of the CRCT (Governor's Office of Student Achievement 2009).

Student successes or lack thereof are to some extent a reflection of the teaching, and it follows that some of the widespread failure of students on these exams is due to a lack of content knowledge on the part of teachers. While Georgia middle and high school teachers are required to pass a content knowledge exam, elementary teachers are not. Over time, there has been a gradual lowering of standards for science teachers in terms of

their science background and content knowledge (Kumar 2003). Even though it has been demonstrated that students who have teachers with a strong science background are more likely to demonstrate learning of key concepts (Anderson 1983), only one third of elementary science teachers have any undergraduate level coursework in critical science classes. Among middle school science teachers, almost half lack in-depth preparation (defined as six or more undergraduate courses) in any science. On one survey designed to assess teacher's perceptions of their own qualifications to teach certain subjects, more than 20% of middle school science teachers indicated that they were not qualified to teach chemistry or physics topics, and 55% of them indicated that they had a moderate or substantial need for professional development of their own content knowledge. These statistics combined led the authors of the report to the conclusion that middle school science teachers are "relatively ill prepared in the sciences" (Weiss et al. 2001).

Gender and Ethnic Identity Affect Success in Science

CRCT scores are also available for specific ethnic populations and gender, and these scores reveal that certain minority populations, as well as females, are less likely to succeed in science. For instance, 53% of black and 44% of Hispanic 8th grade students failed the science portion of the CRCT in 2009, while only 21% of white students did not meet the minimum standard. Gender differences were less stark, but still apparent: 34% of males and 37% of females failed. Previous years' test scores show similar trends. Furthermore, gender differences have been seen in instruments designed to measure content knowledge in teachers, with females performing consistently worse on the assessment than males. Indeed, in one particular study, gender was the best indicator of

success on the instrument, showing higher correlation than other variables chosen for analysis, such as education level and previous courses in science (Kumar and Morris 2005). Similarly, anxiety about college science classes has been demonstrated to exist in females more so than in males (Udo et al. 2005). These disparities have been attributed to many historical, social, and biological factors. For instance, from an historical perspective, science knowledge, since its inception, has been relegated to the realm of the masculine (Watts 2007).

For all students, the distance between "school science," or the content knowledge taught under the national and state curriculums, and "real science," conducted by trained scientists in a spirit of inquiry and according to scientific principles, is vast (Hume and Coll 2010) and might serve to discourage potential future scientists. In an effort to bridge some of this distance, the Bio-Bus program seeks to stimulate inquiry and present students with an opportunity to interact with "real" scientists.

The Bio-Bus Program

The Georgia State University Bio-Bus is a mobile laboratory that travels to schools within Georgia to present curriculum-specific science modules to students in grade levels K-12. Undergraduate and graduate students go in teams to Georgia schools, presenting informative slide shows and guiding the students as they perform simple experiments relevant to the presentation. In this way, young students are given the opportunity to engage in activities that are not otherwise possible in their classroom, and to interact with potential role models who are pursuing careers in the sciences. Since its inception in 1999, the Bio-Bus has visited more than 1200 schools, interacting with more

than 100,000 students. Current module topics include: animal diversity, biotechnology, forensics, chemistry: acids and bases, chemistry: states of matter, geosciences, the heart, microbes, water in the environment, and weather: a class project.

2.2 MATERIALS AND METHODS

Target Identification

After meeting with the program director, administrative director, and lab coordinators, a logic model (Figure 24) was developed using Microsoft PowerPoint to identify the Bio-Bus programs, their desired outcomes, and potential targets for the evaluation program. Using the desired outcomes, areas of interest were selected for both student and teacher evaluations.

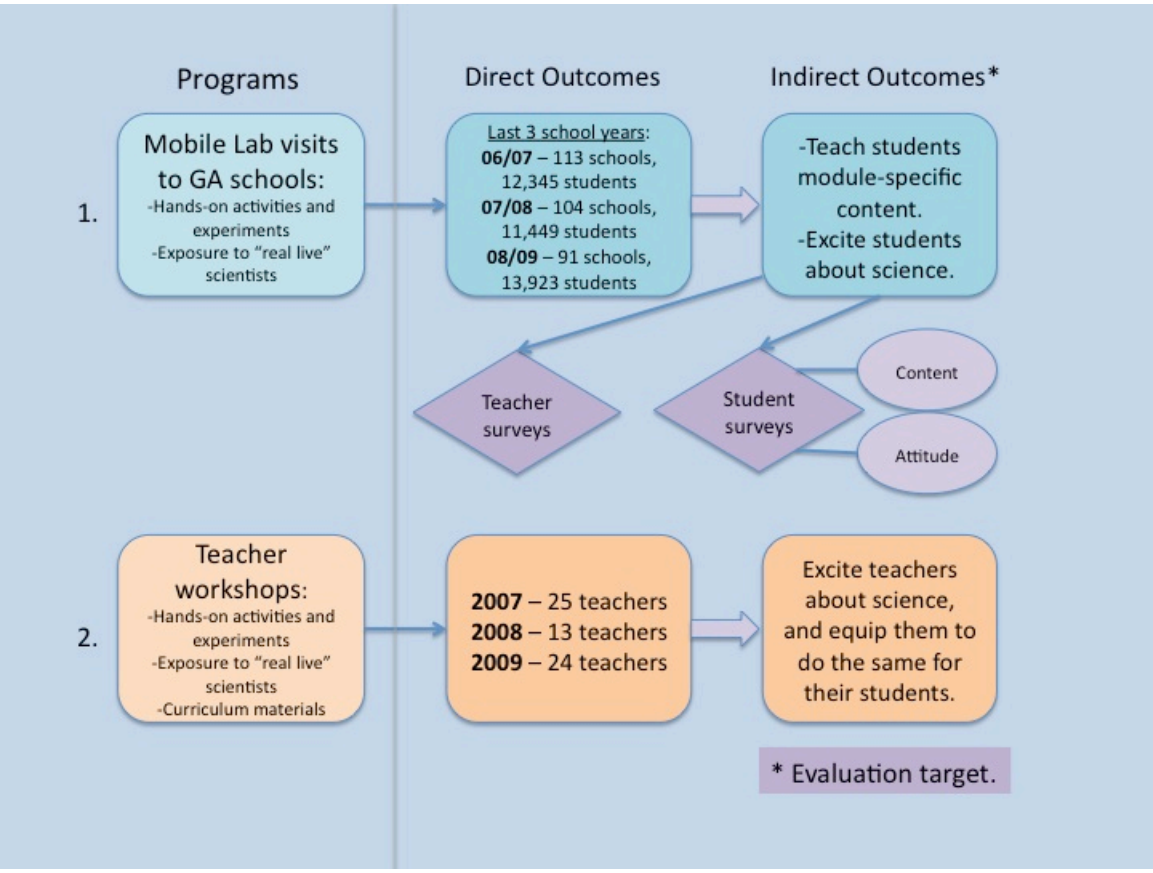

Figure 24. Logic model identifying the activities of the Bio-Bus and their outcomes. The indirect outcomes of the mobile lab visits were selected as targets for the evaluation program. Specifically, student attitudes and content knowledge, as well as teacher attitudes, were identified as areas of interest.

Surveys

Likert scale true/false surveys (see Appendix B for examples) were designed for middle and high school students (grades 6-12) to take before and after the Bio-Bus visit. The surveys included statements pertaining to content knowledge in the particular subject area of the module, statements reflecting the interest of the students, and statements reflecting the confidence of the students; students were directed to circle a letter most representative of their attitudes. Student interest statements included:

- Science is interesting.
- I am interested in science.
- I would like to learn more about science.
- Science is one of my favorite subjects.

Student confidence statements included:

- I think that I could be a scientist.
- I feel like I can understand science.
- I am usually good at science.

Student confidence was also measured by the open-ended question: "In general, do you think that you could be a scientist? Why or why not?" Additionally, during the 2008-2009 school year, statements were included to assess student perception of scientists:

- People who work in a science lab are cool.
- People who teach science are cool.

The surveys were mailed to participating schools, which were selected on the basis of their historical compliance with previous requests made by the Bio-Bus

coordinator. Teachers were asked to administer the pre-visit surveys to their students before the day of the Bio-Bus visit. On the day of the Bio-Bus visit, the teaching fellows administered the post-visit surveys after the presentation of the module. Both pre-visit and post-visit surveys were collected by the Bio-Bus fellows and returned for analysis.

Data Analysis

All survey data were coded and input into Microsoft EXCEL 2008 for Mac (Redmond, WA). The pivot table function was used to obtain numerical results, and the graphing function was used to create all graphs presented. All gaps in student responses or statements in which students circled more than one letter of agreement were coded as blanks, and thus excluded from data analysis. All error bars indicate a p value of 0.05, and thus significance has been defined as any difference greater than five percent.

2.3 RESULTS AND DISCUSSION

Over the course of the study 2,564 surveys were collected and input. Table 11 shows the participating schools and number of surveys administered.

Table 11. Surveys administered to students over the course of the study. Pre-surveys were mailed to participating schools before the Bio-Bus visit, and fellows administered post-surveys on the day of the visit, after completion of the module.

School Year	Pre-visit Surveys	Post-visit Surveys	Target Schools
2006-2007	97	99	Durham Middle
	48	46	Lee Middle
	88	89	Salem Middle
2007-2008	81	52	Columbia High
	71	25	Kennesaw Mtn High
	17	110	Mays High
	100	60	Ola High
	40	34	Columbia Middle
	187	186	SouthCentral Middle
	110	106	Yeager Middle
2008-2009	24	25	Columbia Middle
	26	38	Cross Keys High
	124	123	EaglesLanding Middle
	54	56	East Cobb Middle
	21	15	Torah Day Middle
	200	212	Yeager Middle
TOTAL	**1288**	**1276**	

.

Student Interest

Student interest was measured by responses in Likert scale agreement to the statement, "Science is interesting" before and after the Bio-Bus visit (Figure 25). Likert scale data indicates that 68% of the students that responded (707 out of 1050) had a positive ("true" or "somewhat true") response to the statement before the module while 77% (775 out of 1019) of the students had a positive response to the statement afterwards. In addition to the increase in the percentage of positive responses, both "neutral" and negative ("false" and "somewhat false") responses decreased substantially after the Bio-Bus visit. Presumably, student interest is piqued by the hands-on experiments and demonstrations offered by the Bio-Bus modules, and would therefore serve to increase student interest in science. There were no significant gender differences in response to this statement before or after the Bio-Bus visit. Before the visit, 67.6% (373 out of 552) of females and 67.7% (325 out of 480) of males responded with positive agreement to the statement "Science is interesting." After the Bio-Bus module, 75.6% (385 out of 509) of females and 76.5% (358 out of 468) of males responded positively to the statement.

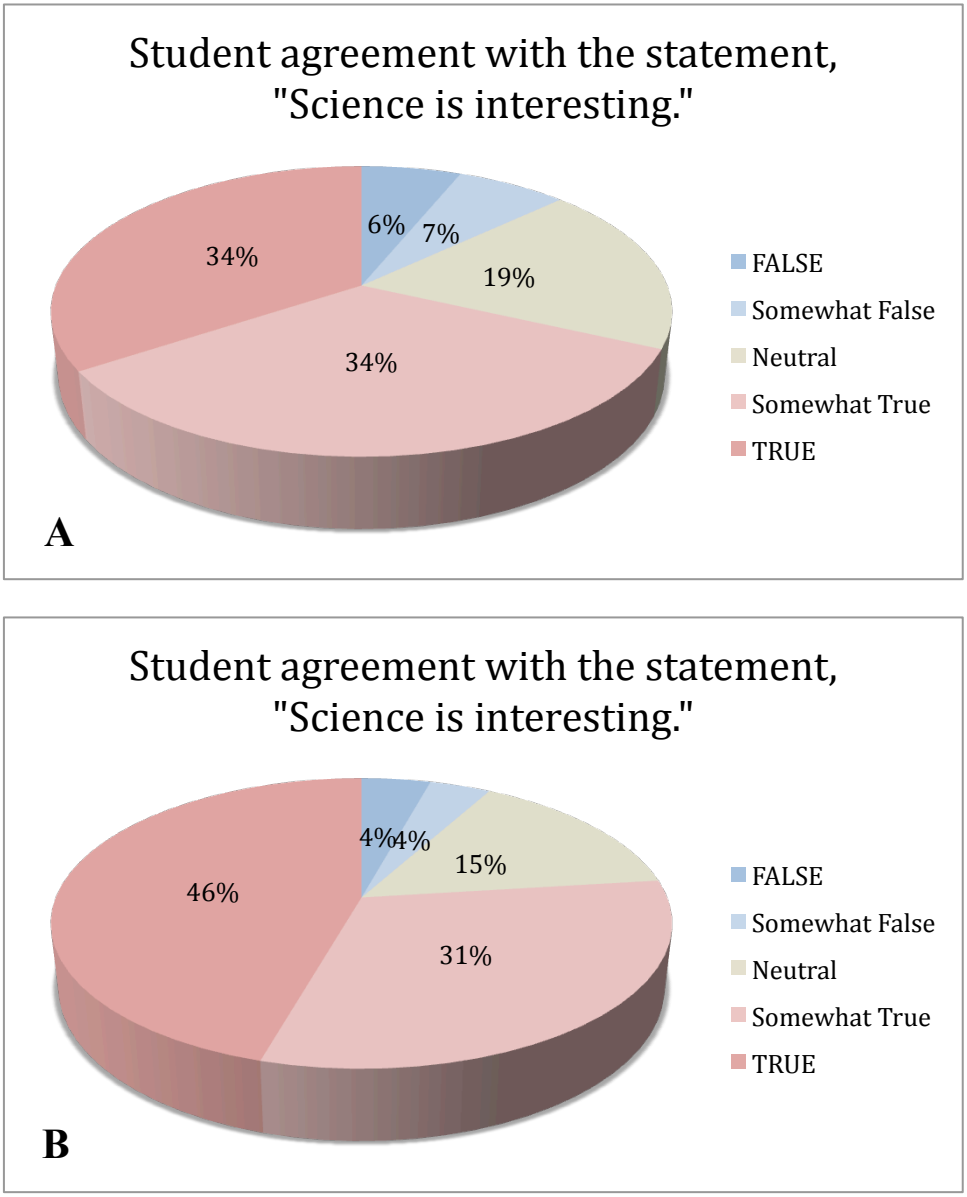

Figure 25. Charts of student agreement with the statement "Science is interesting" before (A) and after (B) the Bio-Bus presentation. Results indicate an increase in agreement with the statement after the module.

Interestingly, data for the very similar statement "I am interested in science" showed a lesser interest both before and after the Bio-Bus visit, but attitude improved with greater percent change after the module. Before the presentation, 43.1% (453 out of 1050) of students indicated positive agreement by selecting "true" or "somewhat true" for this statement; after the module, 56% (591 out of 1019) of students indicated positive agreement. This demonstrates a roughly 30% change over initial student agreement with the statement "I am interested in science." It is possible that the more personal direction "I am…" in the statement causes less student agreement than was seen for the statement "Science is interesting," which might have been perceived as more generic, and therefore easier with which to agree. As with data for that statement, there were no significant gender differences in response to "I am interested in science" either before or after the presentation.

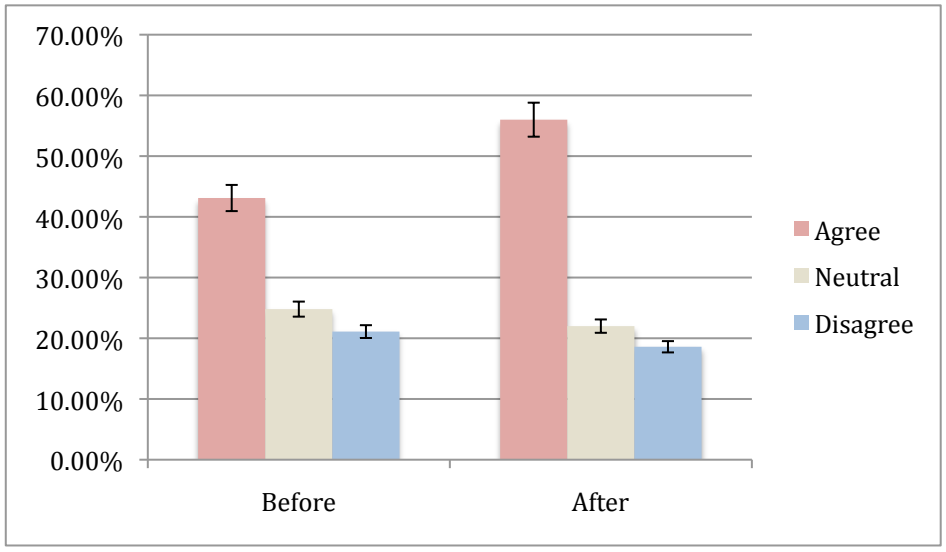

Figure 26. Student agreement with the statement "I am interested in science." Agreement was indicated by students circling "true" or "somewhat true;" neutral was indicated by students circling "neutral;" disagreement was indicated by student circling "false" or "somewhat false." Agreement increased after the Bio-Bus visit.

Student interest was also measured by agreement with the statement, " I would like to learn more about science" (Figure 27). Likert scale data indicates that 50.8% of the students that responded (533 out of 1050) had a positive ("true" or "somewhat true") response to the statement before the module, while 62.1% (633 out of 1019) of the students had a positive response to the statement afterwards. This reflects a 29.9% change in positive agreement with the statement after students experienced the Bio-Bus module. In addition to the increase in the percentage of positive responses, both "neutral" and negative ("false" and "somewhat false") responses decreased substantially after the Bio-Bus visit. While it is impossible to predict from this data, this statement has some level of active participation implied on the part of the student. Further investigation is needed to confirm future student engagement and follow-up on their professed desire to "learn more about science." No significant difference was detected between genders in response to this statement.

The final statement measuring student interest on the surveys was, "Science is one of my favorite subjects." Not surprisingly, student agreement rates with this statement were lower than for any other interest statement, both before and after the Bio-Bus visit, and a large percentage (roughly 34%, or 708 out of 2069 total surveys) of these were left blank. This could be in part due to the fact that this was the last statement on the instrument, and possibly many of the students did not get to those statements. It could also reflect an aversion on the part of the students to responding to this particular statement. Before the Bio-Bus visit, 32.8% of students (344 out of 1050) indicated that science was one of their favorite subjects by circling "true" or "somewhat true," while

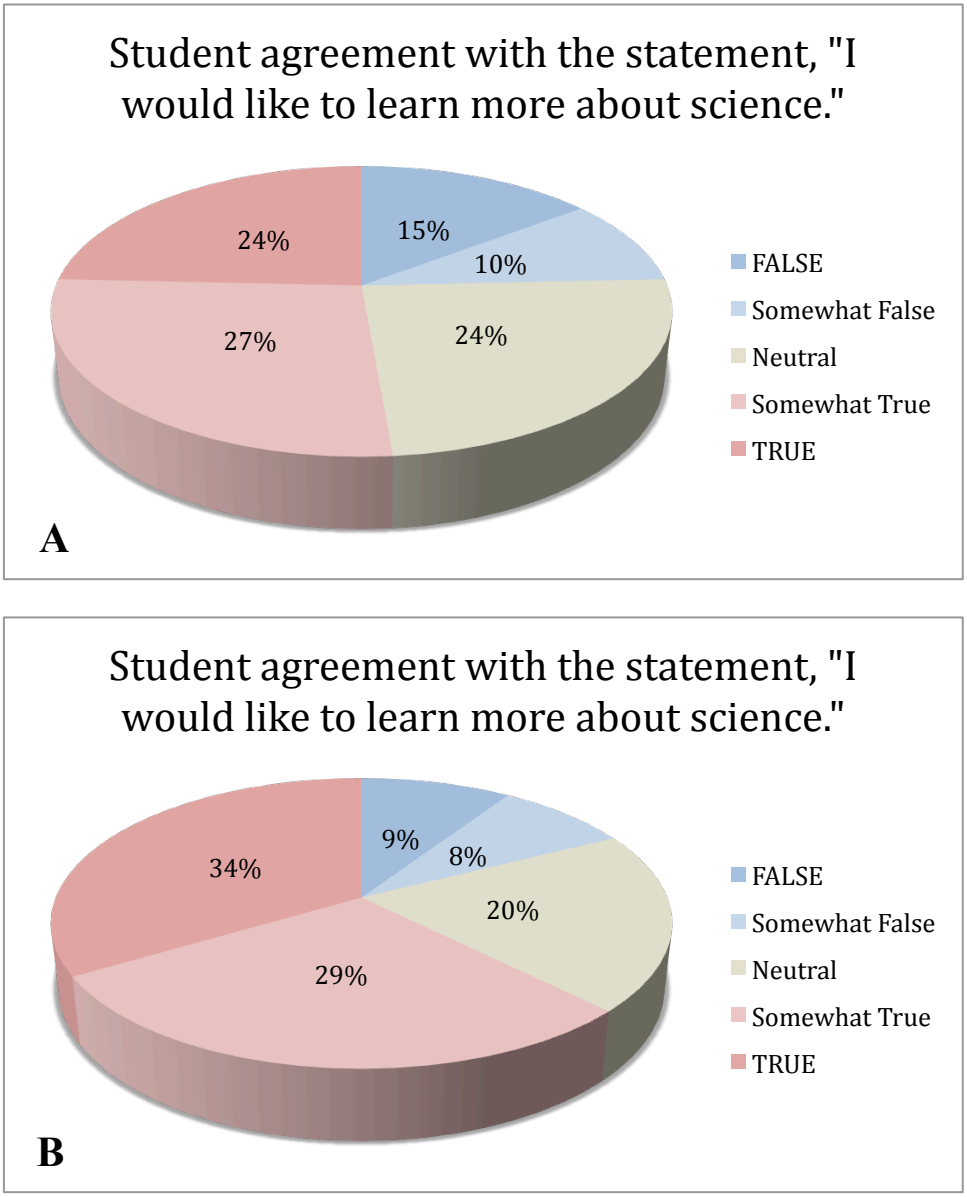

Figure 27. Student agreement with the statement "I would like to learn more about science" both before (A) and after (B) the Bio-Bus presentation. Results indicate an increase in agreement with the statement after the module.

39.9% of students (407 out of 1019) indicated this after the Bio-Bus visit; this reflects a 21.6% change in attitude. As with the rest of the student interest statements, no significant difference was demonstrated between males and females in agreement with the statement, "Science is one of my favorite subjects."

Clearly, the Bio-Bus presentations positively impact student interest in science, with students showing a roughly 20-30% increase in agreement with the statements that reflect their interest in science. Interest in science was consistent between the genders both before and after the Bio-Bus visits, implying perhaps that any substantial difference between male and female performance on standardized tests might not be a result of females being less interested in science. Rather, other variables in attitude might have a greater impact on student performance.

Student Confidence

Less than two thirds of the students chose to answer the open-ended question: "In general, do you think that you could be a scientist?" (1141 pre-visit responses and 959 post-visit responses). This could be partially due to time constraints in finishing the surveys in the allotted amount of time, especially in the case of the post-visit surveys, which were often hurried at the end of the class period after the Bio-Bus visit. Student response to the open-ended question changed substantially after the Bio-Bus visit, indicating that the Bio-Bus positively impacted student confidence (Figure 28). Gender analysis of responses to this question reveal no significant difference in either the starting percentage of girls and boys responding "yes" to this question or the post-visit percentage. In each case, roughly 35% of students indicate that they think they could be scientists before the Bio-Bus visit (163 out of 420 boys responding and 175 out of 501 girls responding); after the visit, 52% of students (199 out of 378 boys responding and 212 out of 402 girls responding) indicate "yes" to this question.

Additionally, data from the Likert-scale statement "I think that I could be a scientist," further confirmed improvement in student confidence following the Bio-Bus visit, with very similar total numbers of student indicating agreement both before (32% of students circled "true" or "somewhat true") and after (49.9% of students circled "true" or "somewhat true") the visit.

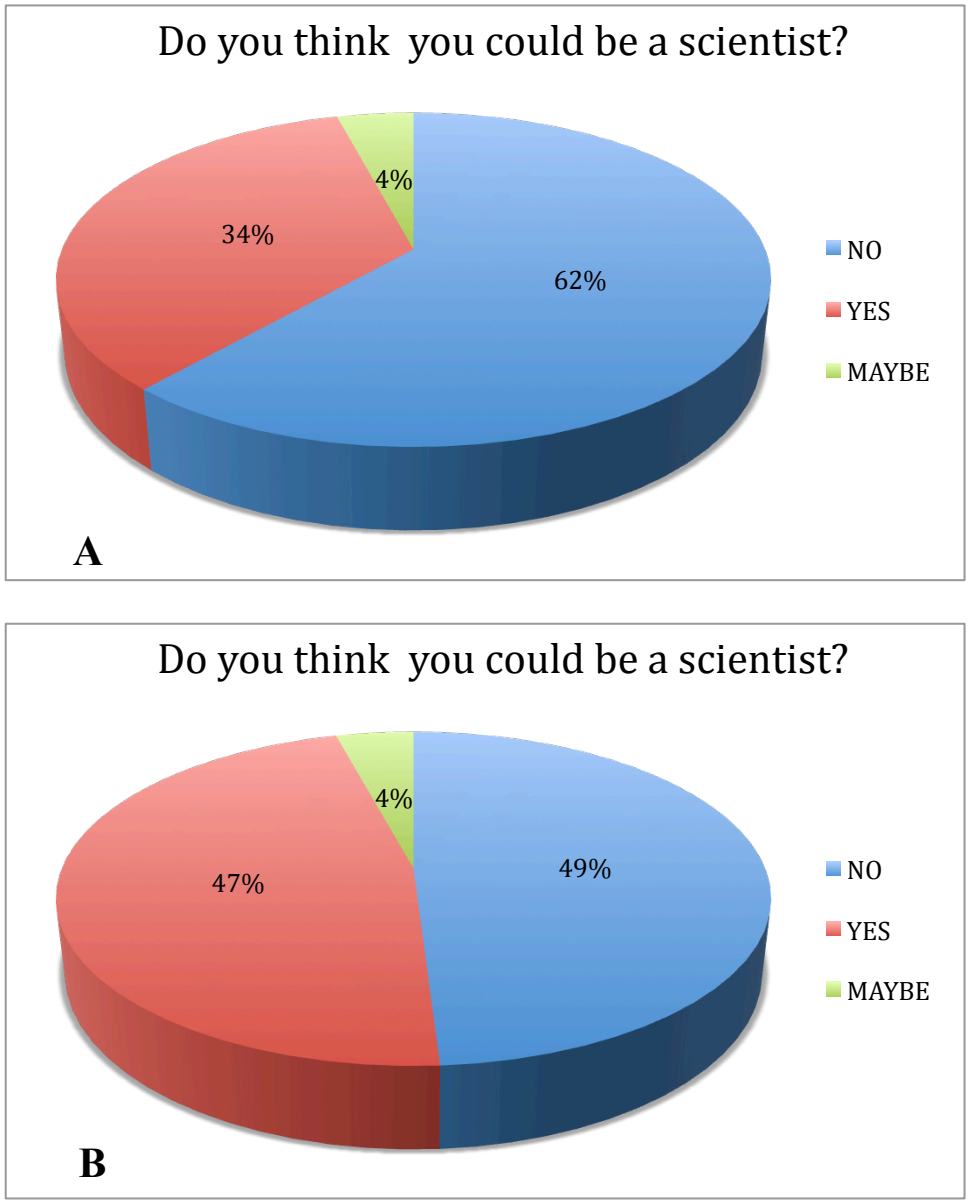

Figure 28. Change in student response to the question "In general, do you think that you could be a scientist?" Chart A shows the percentage of students answering "yes," "no," and "maybe" to this open-ended question before the Bio-Bus visit, while Chart B shows the percentage of students answering "yes," "no," and "maybe" to the question after the Bio-Bus visit. Results indicate an increase in the number of "yes" responses, as well as a decrease in the number of "no" responses.

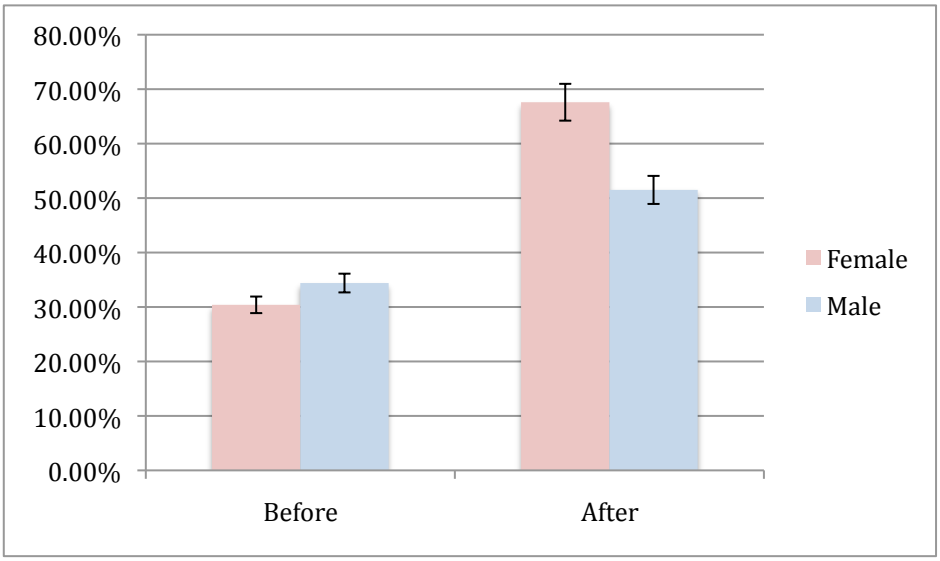

Figure 29. Percentage of males and females indicating "true" or "somewhat true" in agreement with the statement "I think that I could be a scientist."

Interestingly, a gender difference appears in the data obtained from the Likert-scale statement that does not arise in the open-ended question (Figure 29). Of the female students responding to the statement "I think that I could be a scientist," 168 out of 552 circled either "true" or "somewhat true" before the Bio-Bus visit; 344 out of 509 circled either "true" or "somewhat true" after the Bio-Bus visit. Of the male students responding to this statement, 165 out of 480 circled either "true" or "somewhat true" before the Bio-Bus visit; 241 out of 468 circled either "true" or "somewhat true" after the Bio-Bus visit. This could be due in part to the lower numbers of students that answered the open-ended question, potentially allowing a bias that selected for faster students. Presumably, the students that had time to reach the end of the survey were faster readers or more eager to complete the survey, and this subpopulation of students might not demonstrate gender differences. The Bio-Bus clearly has a significant impact on student

response: fully one third more students indicate they think that they could be scientists after having exposure to the Bio-Bus module. While further questioning of students would be necessary to understand the reason for this, it is possible that students are able to identify with the fellows, who are young and diverse, and are thus more likely to envision themselves as future scientists.

Impact on student confidence was also measured with the statement "I feel like I can understand science." Likert scale data indicates that 52% of the students that responded (538 out of 1050) had a positive ("true" or "somewhat true") response to the statement before the module while 69% (695 out of 1019) of the students had a positive response to the statement afterwards. In addition to the increase in the percentage of positive responses, "neutral" and negative ("false" and "somewhat false") responses decreased substantially after the Bio-Bus visit. Many studies have shown that students are more engaged and more likely to succeed in science by doing hands-on activities. Furthermore, 85% (861 out of 1019) of this student population responded with positive agreement ("true" or "somewhat true") to the statement, "I learn better when I do activities than when I just listen in class." Thus, this is a reasonable explanation for the increase in confidence as demonstrated by this data. As students had just completed a module in which they were engaged in learning scientific concepts by doing hands-on experiments, their confidence levels consequently increased.

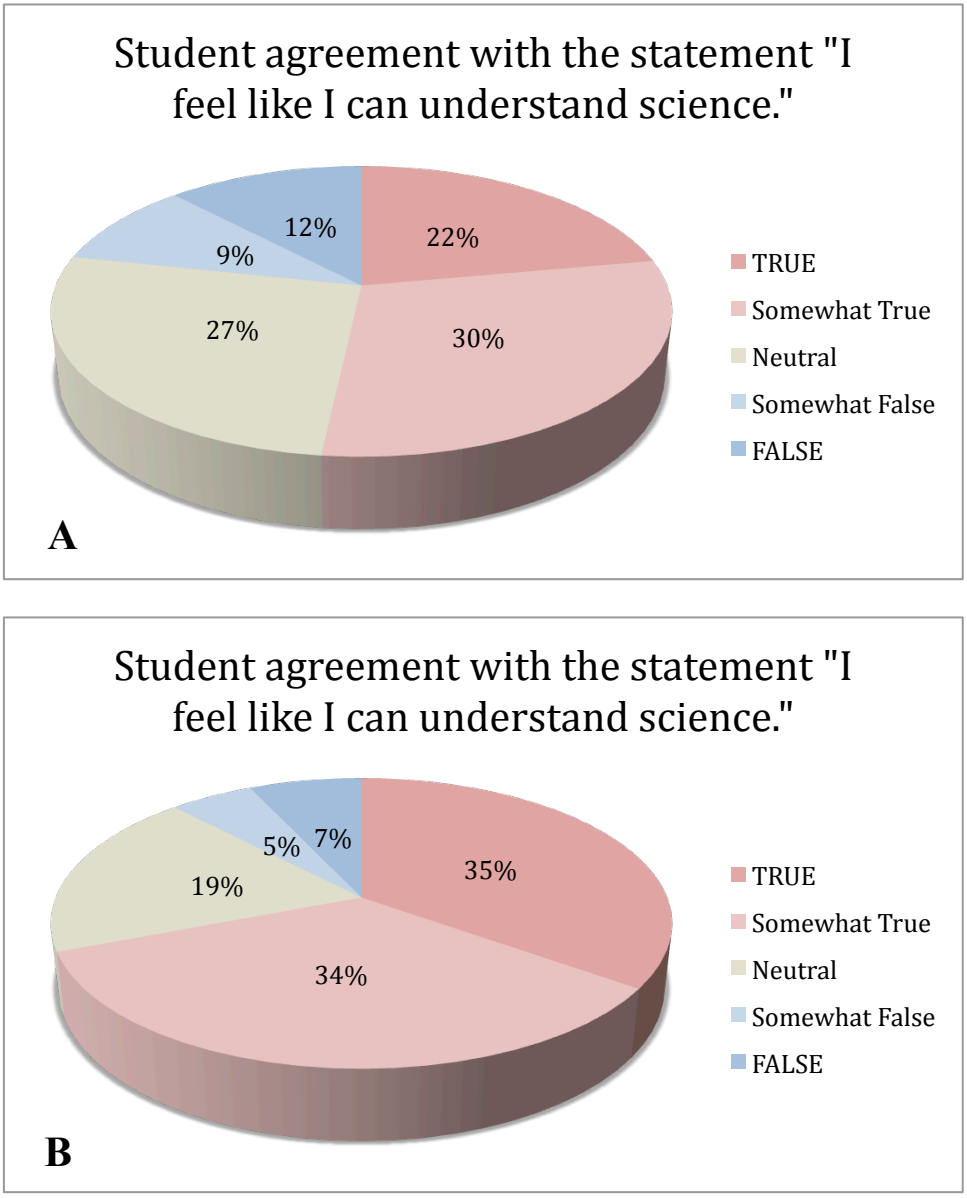

Figure 30. Charts of student agreement with the statement "I feel like I can understand science" before (A) and after (B) the Bio-Bus presentation. Results indicate an increase in agreement with the statement after the module.

Student confidence was further measured by student agreement with the statement, "I am usually good at science." The shift in confidence level measured by this statement did not demonstrate such a significant difference in student response before and after the Bio-Bus visit, with 61.5% responding in positive agreement ("true" or "somewhat true") before the Bio-Bus visit, and 65.7% responding in positive agreement afterwards. Interestingly, there was a substantial gender difference in positive responses to this statement before the Bio-Bus; the difference in responses between the two genders became insignificant after the Bio-Bus visit (Figure 31).

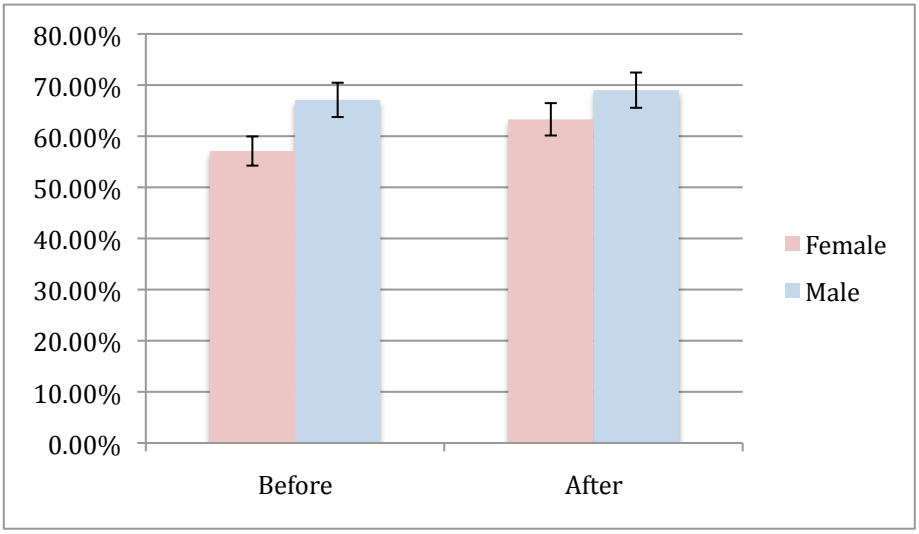

Figure 31. Percentage of males and females indicating "true" or "somewhat true" in agreement with the statement, "I am usually good at science."

It is possible that the use of the modifier "usually" served to temper the change of the responses after the visit, as students most likely thought about their typical performance in science class. This would explain why the response to this statement did not demonstrate the significant change that was demonstrated by the other statements meant to measure confidence level of the students.

As with the data presented on student interest, the Bio-Bus visits to schools clearly impact student confidence in a positive way. Some significant gender differences were noted in the area of student confidence, and this area of attitude might be a candidate for partial explanation of gender differences in standardized test scores. While there were some gender differences noticed in the area of student confidence, the Bio-Bus seems to remedy that difference, at least temporarily.

Student Perception of Scientists

Student perception of scientists was measured, as it is hypothesized that the idea that scientists are "nerdy" might pose a barrier to positive attitude towards science. As these statements were not included in the survey until the 2008-2009 school year, there were fewer student responses (215 pre-surveys and 231 post-surveys) included in this analysis. The Bio-Bus visit had a significant impact on student agreement with the statement, "People who work in a science lab are cool." Before the visit, 32.6% of students (70 out of 215) agreed with this statement, circling either "true" or "somewhat true" on their survey. After the visit, 48.1% of students (111 out of 231) agreed with this statement, demonstrating an almost 50% increase in agreement. This substantially large change in attitude can be attributed to the exposure that students get to "real, live" scientists, or teaching fellows. Presumably, their stereotypical idea of "nerdy scientist" could be replaced through their encounter with the teaching fellows, all of which are extremely "cool."

Interestingly, this change seemed to occur to a greater extent in females than in males (Figure 32). Before the visit, 33.1% of females and 31.5% of males indicated

agreement with the statement, while 50.5% of females and 40.2% of males indicated agreement exposure to the Bio-Bus fellows.

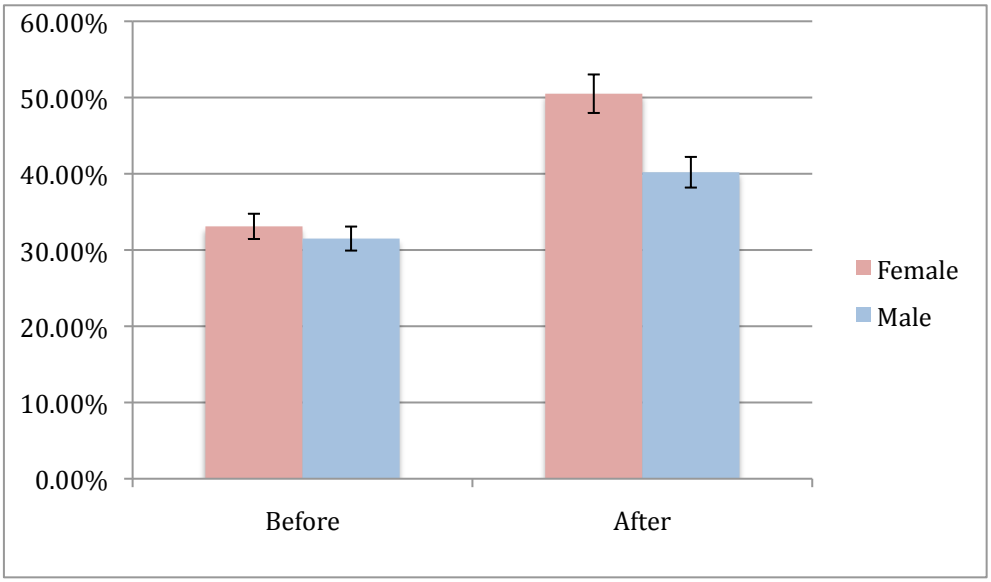

Figure 32. Student agreement with the statement, "People who work in a science lab are cool" before and after the Bio-Bus visit. While students of both genders showed greater agreement with the statement, females were much more positively impacted than males.

Similarly, students were asked to rate agreement with the statement, "People who teach science are cool" as a measure of perception of scientists. Interestingly, the percent change in student agreement with this statement was a barely significant 8.6%. Before the visit, 39.5% of students (85 out of 215) agreed, by circling "true" or "somewhat true," that science teachers are "cool," while 42.9% of the students (99 out of 231) indicated this after the Bio-Bus visit. Perhaps the students did not consider the Bio-Bus fellows to be "people who teach science," but rather people who "do" science, who just happened to be teaching during this encounter. Regardless of this inconsistency, the Bio-Bus clearly has a positive impact on students' perception of scientists. These improvements in

student perception of scientists after meeting actual scientists is consistent with other published studies of this phenomenon (Flick 1990; Bodzin and Gehringer 2001).

Summary and Future Studies

All findings indicate that the Bio-Bus is achieving its goals of positively impacting student attitude towards science. This is demonstrated by positive changes in agreement with statements targeting areas of student interest, student confidence, and student perception of scientists. In addition to the direct impact that the Bio-Bus has on student attitude, it is possible that the modeling of inquiry-based teaching methods, primarily through hands-on approaches to learning, might positively impact teachers to some extent. For many reasons, teachers have difficulty implementing inquiry-based methods (Jones and Eick 2007), and thus exposing teachers to these methods might have a more extensive impact on students if teachers are encouraged to make a shift towards this approach.

While the findings are positive, the most obvious limitation to the study is in the area of retention of impact; as the surveys were given to the students immediately after the Bio-Bus encounter, there is no way to know if the Bio-Bus has any long-term effects on student attitude. The simplest way to remedy this gap would be administration of another post-visit survey, possibly weeks or months after the module. Additionally, qualitative data might provide another depiction of the impact of the Bio-Bus on student attitude. Student interviews or focus groups might help to answer questions as to why student attitude improves with the Bio-Bus visit. Finally, only a select few realms of student attitude were targeted for assessment by these surveys. Additional statements

that examine student attitudes towards the relevance of science to their lives, for instance, might be included on future surveys.

REFERENCES

Abd, H., T. Johansson, I. Golovoliov, G. Sandstrom, and M. Forsman. 2003. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl. Env. Microbiol. **69**: 600-606.

Alizadeh, H., S. Neelam, M. Hurt, and J.Y. Niederkorn. 2005. Role of contact lens wear, bacterial flora, and mannose-induced pathogenic protease in the pathogenesis of amoebic keratitis. Infect. Immun. **73**: 1061-1068.

Anderson, R.D. 1983. A consolidation and appraisal of science meta-analyses. J. Res. Sci. Teaching **20**: 479-509.

Anderson, I.J., R.F. Watkins, J. Samuleson, D.F. Spencer, W.H. Majoros, M.W. Gray, and B.J. Loftus. 2005. Gene discovery in the *Acanthamoeba castellanii* genome. Protist **156**: 203-214.

Arif, I.A., M.A. Bakir, H.A. Khan, A.H. Al Farhan, A.A. Al Homaidan, A.H. Bahkali, M.A. Sadoon, M. Shobrak. 2010. A brief review of molecular techniques to assess plant diversity. Int. J. Mol. Sci. **11**: 2079-2096.

Aufauvre-Brown, A., J. Cohen, and D.W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. **30**: 2991-2993.

Awaad, S.T, W.M. Petroll, J.P. McCulley, and H.D. Cavanagh. 2007. Updates in *Acanthamoeba* keratitis. Eye Contact Lens **33**:1-8.

Barett, R.A. and M. Alexander. 1977. Resistance of cyst of amoebae to microbial decomposition. Appl. Env. Microbiol. **33**: 670-674.

Bodzin, A., and M. Gehringer. 2001. Breaking science stereotypes: can meeting actual scientists change students' perceptions of scientists? Sci. and Child. **38**: 24-27.

Bohle, K., A. Jungebloud, Y. Gocke, A. Dalpiaz, C. Cordes, H. Horn, and D.C. Hempel. 2007. Selection of reference genes for normalization of specific gene quantification data of *Aspergillus niger*. J. Biotech. **132**: 353-358.

Booton, G.C., D.J. Kelly, Y.W. Chu, D.V. Seal, E. Houang, D.S.C. Lam, T.J. Byers, and P.A. Fuerst. 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens case, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. J. Clin. Microbiol. **40**: 1621-1625.

Bowers, B., and E.D. Korn. 1969. The fine structure of *Acanthamoeba castellanii* (Neff Strain). II. Encystment. J. Cell Biol. **41**: 786-805.

Brown, M.R., and J. Barker. 1999. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. Trends Microbiol. **7**: 46-50.

Buck, S.L., R.A. Rosenthal, and B.A. Schlech. 2000. Methods used to evaluate the effectiveness of contact lens care solutions and other compounds against *Acanthamoeba*: a review of the literature. CLAO J. **26**: 72-84.

Cirillo, J.D., S.L.G. Cirillo, L. Yan, L.E. Bermudez, S. Falkow, and L.S. Thomkins. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. Infect. Immun. **67**: 4427-4434.

Cirillo, J.D., S. Falkow, L.S. Tompkins, and L.E. Bermudez. 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. Infect. Immun. **65**: 3759-3767.

Clarholm, M. 2002. Bacteria and protozoa as integral components of the forest ecosystem – their role in creating a naturally varied soil fertility. Antonie Van Leeuwenhoek **81**: 309-318.

Clarke, D.W and J.Y. Niederkorn. 2006. The pathophysiology of *Acanthamoeba* keratitis. TRENDS Parasitol. **22**: 175-180.

Cohen, E. 2009. Contact lens solutions: part of the problem. Arch. Opthamol. **127**: 1544-1546.

Cordingley, J.S., R.A. Wills, and C.L. Villemez. 1996. Osmolarity is an independent trigger of *Acanthamoeba castellanii* differentiation. J. Cell Biochem. **61**: 167-171.

Corsaro, D. and D. Venditti. 2010. Phylogenetic evidence for a new genotype of Acanthamoeba (Amoebozoa, Acanthamoebida). Parasitol. Res. [Epub ahead of print].

Dart, J.K., V.P Saw, and S. Kilvington. 2009. *Acanthamoeba* keratitis: diagnosis and treatment update 2009. Am. J. Opthamol. **148**: 487-499.

De Jonckheere, J.F. 1991. Ecology of *Acanthamoeba*. Rev. Infect. Dis. **13**: 385-387.

Donzis, P.B., B.J. Mondino, B.A. Weissan, and D.A. Brucker. 1989. Microbial contamination of contact lens cases in the west of Scotland. Br. J. Ophthalmol. **77**: 41-45.

Dudley, R., S. Alsam, and N.A. Khan. 2007. Cellulose biosynthesis pathway is a potential target in the improved treatment of *Acanthamoeba* keratitis. Appl. Microbiol. Biotechnol. **75**: 133-140.

Dudley, R., S. Alsam, and N.A. Khan. 2008. The role of proteases in the differentiation of *Acanthamoeba castellanii*. FEMS Microbiol. Lett. **286**: 9-15.

Dudley, R., E.L. Jarroll, and N.A. Khan. 2009. Carbohydrate analysis of *Acanthamoeba castellanii*. Exp. Parasitol. **122**: 338-343.

Dudley, R., A. Matin, S. Alsam, J. Sissons, A.H. Maghsood, and N.A. Khan. 2005. Acanthamoeba isolates belonging to T1, T2, T3, T4 but not T7 encyst in response to increased osmolarity and cysts do not bind to human corneal epithelial cells. Acta Tropica **95**: 100-108.

Feng, Y., Y. Hsiao, H. Chen, C. Chu, P. Tang, and C. Chiu. 2009. Apoptosis-like cell death induced by Salmonella in *Acanthamoeba rhysodes*. Genomics **94**: 132-137.

Flick, I. 1990. Scientists in residence program: improving children's images of science and scientists. School Sci. Math. **90**: 204-214.

Furuhata, K., K. Ogihara, R. Okuno, K. Oonaka, and M. Fukuyama. 2009. Growth in *Acanthamoeba sp*. and antibiotic susceptibility of *Legionella micdadei* isolated from hot spring water samples. Biocontrol. Sci. **14**: 181-184.

Gao, L.Y. and Y.A. Kwaik. 2000. The mechanism of killing and exiting the protozoan host *Acanthamoeba polyphaga* by *Legionella pneumophila*. Env. Micro. **2**: 79-90.

Garate, M., Z. Cao, E. Bateman, and N. Panjwani. 2004. Cloning and characterization of a novel mannose-binding protein of *Acanthamoeba*. J. Biol. Chem. **279**: 29849-29856.

Garate, M., I. Cubillos, J. Marchant, and N. Panjwani. 2005. Biochemical characterization and functional studies of *Acanthamoeba* mannose-binding protein. Inf. Immun. 73: 5775-5781.

Georgia Department of Education. www.doe.k12.ga.us. Data downloaded April 2010.

Governor's Office of Student Achievement. 2009 Report Card. http://reportcard2009.gaosa.org Report downloaded April 2010.

Greub, R.J. and D. Raoult. 2004. Microorganisms resistant to free-living amoebae. Clin. Microbiol. Rev. **17**: 413-433.

Guerra, J.C., M.R. Issa, F.E. Carneiro, R. Strapazzon, and G. Moretto. 2010. RAPD identification of Varroa destructor genotypes in Brazil and other regions of the Americas. Genet. Mol. Res. **9**: 303-308.

Harb, O.S., L.Y. Gao, and Y.A. Kwaik. 2000. From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. Environ. Microbiol. **2**: 251-265.

Heinz, E., I. Kolarov, C. Kastner, E.R. Toenshoff, M. Wagner, and M. Horn. 2007. An *Acanthamoeba* sp. containing two phylogenetically different bacterial endosymboints. Environ. Microbiol. **9**: 1604-1609.

Hirukawa, Y., H. Nakato, S. Izumi, T. Tsuruhara, and S. Tomino. 1998. Structure and expression of a cyst specific protein of *Acanthamoeba castellanii*. Biochim. Biopys. Acta. **1398**: 47-56.

Hughes, R., W. Heaselgrave, and S. Kilvington. 2003. *Acanthamoeba polyphaga* strain age and method of cyst production influence the observed efficacy of therapeutic agents and contact lens disinfectants. Antimicrob. Agents Chemother. **47**: 3080-3084.

Hugo, E.R., W.R. McLaughlin, K. Oh, and O.H. Tuovinen. 1991. Quantitative enumeration of *Acanthamoeba* for evaluation of cyst inactivation in contact lens care solutions. Inv. Ophth. Vis. Sci. **32**: 655-657.

Hume, A. and R. Coll. 2010. Authentic student inquiry: the mismatch between the intended curriculum and the student-experienced curriculum. Res. Sci. Tech. Ed. **28**: 43-62.

Jones, M.T., and C.J. Eick. 2007. Providing bottom-up support to middle school science teachers' reform efforts using inquiry-based kits. J. Sci. Teacher Educ. **18**: 913-934.

Joslin, C.E., E.Y. Tu, M.E. Shoff, G.C. Booton, P.A Feurst, T.T. McMahon, R.J. Anderson, M.S. Dworkin, J. Sugar, F.G. Davis, and L.T. Stayner. 2007. The association of contact lens solution use and *Acanthamoeba* keratitis. Am. J. Ophthalmol. **144**: 169-180.

Khan, N.A. 2009. *Acanthamoeba*: biology and pathogenesis. Caister Academic Press, Norfolk.

Kilvington, S. 1990. Activity of water biocide chemicals and contact lens disinfectants on pathogenic free-living amoebae. Internat. Biodeterior. **26**: 127-138.

Kilvington, S., J.R. Beeching, and D.G. White. 1991. Differentiation of *Acanthamoeba* strains from infected corneas and the environment by using restriction endonuclease digestion of whole-cell DNA. J. Clin. Microbiol. **29**: 310-314.

Kohsler, M., D. Leitsch, U. Furnkranz, M. Duchene, H. Aspock, and J. Walochnik. 2008. *Acanthamoeba* strains lose their abilities to encyst synchronously upon prolonged axenic culture. Parisitol. Res. **102**: 1069-1072.

Kong, H.H., J.Y. Shin, H.S. Yu, J. Kim, T.W. Hahn, Y.H. Hahn, and D.I. Chung. 2002. Mitochondrial DNA restriction fragment length polymorphism (RFLP) and 18S small-subunit ribosomal DNA PCR-RFLP analyses of *Acanthamoeba* isolated from contact lens storage cases of residents in southwestern Korea. J. Clin. Microbiol. **40**: 1199-1206.

Kumar, D.D. 2003. Trends in post-secondary science in the United States. Ann. Am. Acad. Pol. Soc. Sci. **585**: 124-133.

Kumar, D.D, and J.D. Morris. 2005. Predicting scientific understanding of prospective elementary teachers: role of gender, education level, courses in science, and attitudes toward science and mathematics. J. Sci. Ed. Tech. **14**: 387-391.

Larkin, D.F., M. Berry, and D.L. Easty. 1991. In vitro corneal pathogenicity of *Acanthamoeba*. Eye **5**: 560-568.

Leitsch, D., M. Kohsler, M. Marchetti-Deschmann, A. Deutsch, G. Allmaier, M. Duchene, and J. Walochnik. 2010. Major role for cysteine proteases during the early phase of *Acanthamoeba castellanii* encystment. Eukaryot. Cell **9**: 611-618.

Lloyd, D., N.A. Turner, W. Khunkitti, A.C. Hann, J.R. Furr, and A.D. Russell. 2001. Encystation in *Acanthamoeba castellanii*: development of biocide resistance. J. Eukaryot. Microbiol. **48**: 11-16.

Lonnen, J., W. Heaselgrave, M. Nomachi, O. Mori, and J. Santodomingo-Rubido. 2010. Disinfection efficacy and encystment rate of soft contact lens multipurpose solutions against *Acanthamoeba*. Eye Contact Lens **36**: 26-32.

Lorenzo-Morales, J., J. Kliescikova, E. Martinez-Carretero, L.M. dePablos, B. Profotova, E. Nohynkova, A. Osuna, and B.Valladares. 2008. Glycogen phosphorylase in *Acanthamoeba spp*.: determining the role of the enzyme during the encystment process using RNA interference. Eukaryotic Cell **7**: 509-517.

Lorenzo-Morales, J., A. Ortega-Riveras, P. Foronda, N. Abreu-Acosta, D. Ballart, E. Martinez, and B. Valladares. 2005. RNA interference (RNAi) for the silencing of extracellular serine proteases genes in *Acanthamoeba*: molecular analysis and effect on pathogenicity. Mol. Biochem Parasitol. **144**: 10-15.

Makioka, A., M. Kumagai, H. Ohtomo, S. Kobayashi, and T. Takeuchi. 2000. Effect of the antitubulin drug oryzalin on the encystation of *Entabmoeba invadens*. Parasit. Res. **86**: 625-629.

Makioka, A., M. Kumagai, H. Ohtomo, S. Kobayashi, and T. Takeuchi. 2001. Inhibition of encystation of *Entaboeba invadens* by wortmannin. Parasit. Res. **87**: 371-375.

Marciano-Cabral, F. and G. Cabral. 2003. *Acanthamoeba* spp. as agents of disease in humans. Cli. Microbiol. Rev. **16**: 273-307.

Martin-Navarro, C., A. Lopez-Arencibia, J. Lorenzo-Morales, S. Oramas-Royo, R. Hernandez-Molina, A. Estevez-Braun, A. Ravelo, B. Valladares, and J. Pinero. 2010. *Acanthamoeba castellanii* Neff: In vitro activity against the trophozoite stage of a natural sesquiterpene and a synthetic cobalt(II)-lapachol complex. Exp. Parasitol. Epub ahead of print.

Martinez, A.J. 1985. Free-living amoebas: natural history, prevention, diagnosis, pathology and treatment of disease. CRC Press, Boca Raton: 5-42.

Massachusetts Medical Society. 1986. *Acanthamoeba* keratitis associated with contact lenses – United States. Morb. Mort. Week. Rep. **35**: 405-408.

Mazur, T., E. Hadas, and I. Iwanicka. 1995. The duration of the cyst stage and the viability and virulence of *Acanthamoeba* isolates. Trop. Med. Parasitol. **46**: 106-108.

McClellan, K., K. Howard, E. Mayhew, J. Niederkorn, and H. Alizadeh. 2002. Adaptive immune responses to *Acanthamoeba* cysts. Exp. Eye Res. **75**: 285-293.

Moon, E., D. Chung, Y. Hong, T. Ahn, and H. Kong. 2008a. *Acanthamoeba castellanii:* gene profile of encystation by ESTs analysis and KOG assignment. Exp. Parasit. **119**: 111-116.

Moon, E., D. Chung, Y. Hong, and H. Kong. 2007. Differentially expressed genes of *Acanthamoeba castellanii* during encystation. Korean J. Parasitol. **45**: 283-285.

Moon, E., D. Chung, Y. Hong, and H. Kong. 2008b. Characterization of a serine proteinase mediating encystation of *Acanthamoeba*. Eukary. Cell **7**: 1513-1517.

Moon, E., D. Chung, Y. Hong, and H. Kong. 2009. Autophagy protein 8 mediating autophagosome in encysting *Acanthamoeba*. Mol. Biochem. Parasitol. **168**: 43-48.

Moon, E., J.O. Kim, Y.H. Xua, Y.S. Yun, S.W. Kang, Y.S. Lee, T.I. Ahn, Y.C. Hong, D.I. Chung, and H.H. Kong. 2009. Construction of EST database for comparative gene studies of *Acanthamoeba*. Korean J. Parasitol. **47**: 103-107.

Moraes, J. and S.C. Alfieri. 2008. Growth, encystment and survival of *Acanthamoeba castellanii* grazing on different bacteria. FEMS Microbiol. Ecol. **66**: 221-229.

Neff, R.J., S.A. Ray, W.F. Benton, and M. Wilborn. 1964. Induction of synchronous encystment (differentiation) in *Acanthamoeba sp*. In Methods in Cell Physiology. D.M. Prescott, editor. Academic Press Inc., New York: 1-55.

Price, C., S. Al-Khodor, T. Al-Quadan, M. Santic, F. Habyarimana, A. Kalia, and Y. Kwaik. 2009. Molecular mimicry by and F-box effector of *Legionella pneumophila* hijacks a conserved polyubiquitination machinery within macrophages and protozoa. 2009. PloS Pathog. **5**: e1000704.

Qureshi, M.N., A.A. Perezll, R.M. Madayag, and E.J. Bottone. 1993. Inhibition of *Acanthamoeba* species by *Pseudomonas aeruginosa*: rationale for their selective exclusion in corneal ulcers and contact lens care systems. J. Clin. Microbiol. **6**:1908-1910.

Schmitz-Esser, S., E.R. Toenshoff, S. Haider, E. Heinz, V.M. Hoenninger, M. Wagner, and M. Horn. 2008. Diversity of bacterial endosymbionts of environmental *Acanthamoeba* isolates. Appl. Env. Microbiol. **74**: 5822-5831.

Schroeder, J.M, G.C. Booton, J. Hay, I.A. Niszl, D.V. Seal, M.B. Markus, P.A. Fuerst, and T.J. Byers. 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. J. Clin. Microbiol. **39**: 1903-1911.

Sharma, S., P. Garg, and G.N. Rao. 2000. Patient characteristics, diagnosis, and treatment of non-contact lens related *Acanthamoeba* keratitis. Br. J. Opthalmol. **84**: 1103-1108.

Sriram, R., M. Shoff, G. Booton, P. Fuerst, and G.S. Visvesvara. Survival of *Acanthamoeba* cysts after desiccation for more than 20 years. J. Clin. Microbiol. **46**: 4045-4048.

Stehr-Green J.K., T.M. Bailey, T.H. Brandt, J.H. Carr, W.W. Bond, and G.S. Visvesvara. 1987. *Acanthamoeba* keratitis in soft contact lens wearers, a case control study. J. Am. Med. Assoc. **258**: 57-60.

Stehr-Green J.K., T.M. Bailey, and G.S. Visvesvara. 1989. The epidemiology of *Acanthamoeba* keratitis in the United States. Am. J. Opthamol. **107**: 331-336.

Stratford, M.P. and A.J. Griffiths. Variations in the properties and morphology of cysts of *Acanthamoeba castellanii*. J. Gen. Microbiol. **108**: 33-37.

Tu, E.Y., C.E. Joslin, J. Sugar, M.E. Shoff, and G.C. Booton. 2008. Prognostic factors affecting visual outcome in *Acanthamoeba* keratitis. Ophthalmol. **115**: 1998-2003.

Turner, N.A., A.D. Russell, J.R. Furr, and D. Lloyd. 2000. Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. J. Antimicrob. Chemo. **46**: 27-34.

Udo, M.K., G.P. Ramsey, and J.V. Mallow. 2005. Science anxiety and gender in students taking general education science courses. J. Sci. Ed. Tech. **13**: 435-446.

Wang, X., and D.G. Ahearn. 1997. Effect of bacteria on survival and growth of *Acanthamoeba castellanii*. Curr. Microbiol. **34**: 212-215.

Watts, R. 2007. Whose knowledge? Gender, education, science and history. His. Ed. **36**: 283-302.

Weisman, R.A. 1976. Differentiation in *Acanthamoeba castellanii*. Ann. Rev. Microbiol. **30**: 189-219.

Weisman, R.A., R.S. Spiegel, J.G. McCauley. 1970. Differentiation in *Acanthamoeba*: glycogen levels and glycogen synthase activity during encystment. Biochim. Biophys. Acta **201**: 45-53.

Weiss, I.R., E.R. Banilower, K.C. McMohan, and P.S. Smith. 2001. Report of the 2000 national survey of science and mathematics education. Horizon Research Inc.: Research Triangle Park, NC.

Yang, Z., Z. Cao, and N. Panjwani. 1997. Pathogenesis of *Acanthamoeba* keratitis: carbohydrate-mediated host-parasite interactions. Infect. Immun. **65**: 439-445.

Yang, S. and C. Villemez. 1994. Cell surface control of differentiation in *Acanthamoeba*. J. Cell. Biochem. **56**: 592-596.

Zaman, V., M. Zaki, and M. Manzoor. 1999. A*canthamoeba* in human faeces from Karachi. Ann. Trop. Med. Parasitol. **93**: 189-191.

Zhao, G., S. Sun, J. Zhao, L. Xie. 2010. Genotyping of *Acanthamoeba* isolates and clinical characteristics of patients with *Acanthamoeba* keratitis in China. J. Med. Microbiol. Epub ahead of print.

APPENDIX A: DNA's sequenced for this study

Gene	Sequence	Accession no.
A. castellanii, 18S partial sequence	CTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAA TGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGT CAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTG CGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAAC GAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAG TCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACG TTGAATACAAAACACCACCATCGGCGCGGTCGTCCTTGG CGTCGGTCCTTCACGGGGCCGGCGCGAGGGCGGCTTAGC CCGGTGGCACCGGTGAATGACTCCCCTAGCAGCTTGTGA GAAATCATAAGTCTTTGGGTTCCGGGGGGAGTATGGTCG CAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCAC CAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGG GAAACTTACCAGGTCCGGACATAGTAAGGATTGACAGAT TGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGC CGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGT	100% homology with U07413
A. polyphaga, 18 S partial sequence	CCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGG GTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTA ATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAAC TTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAA GAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTC GTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGA GACGTTGAATACAAAACACCACCATCGGCGCGGTCGTCC TTGGCGTCGGTCCTTCACGGGGCCGGCGCGAGGGCGGCT TAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCAGCTT GTGAGAAATCATAAGTCTTTGGGTTCCGGGGGGAGTATG GTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCA CCACCGGGAGTGGAGCCTGCGGCTTAATTTGACTCAACA CGGGGAAACTTACCAGGTCCGGACATGATAAGGATTGAC AGATTGATAGCTCTTTCTTGATTCTGTGGGTGGTGGTGCA TGGCCGTTC	100% homology with U07413
A. castellanii, enolase partial sequence	GGGCGTGATGGTGTCGCACAGGAGCGGTGAGACCGAGGA CACCTTCATCGCTGACCTCGTGGTCGGCCTCGGCACCGGC CAGATCAAGACCGGCGCCCCGTGCAGGTCCGAGAG	89% homology with AB025330
A. castellanii subtilisin-like serine proteinase partial sequence	CCGTGGTCGACGCTGGCGTGCCCTTCGCCGTCGCCGCCGG TAACGAGGACCAGAACGCGTGCAACACCAGCCCGGCCTC GGAGGCCAAGGCCATCACCGTGGGCGCCACCTACTTCAA GGGCAACTACACCCAGGACACCCGCGCCTACTTCTCCAA CTGGGGCACCTGCGTCGACATCCTGGCGCCCGGCCAGTC GATCAAGTCGG	100% homology with EU365402
A. polyphaga glycogen posphorylase partial sequence	CTACCCGGCCTGGGGCTACGGCCTGCGCTACACCTACGG CATCTTCACGCAGAAGGTGGTCGACGGCTACCAGGTGGA GACCGCTGACGCGTGGCTCACCGGCGGCAACGGCTACCC GTGGGAGGTGGAGCGCAAGGACGTCGTCTACCCGGTGCG CTTCTACGGCGAGGTGATCCAGGTGGGCTACAAGAAGTA CAAGTGGACCGGCGGCGAGGTGGTCATGGCGCAGGCCTA	100% homology with EU273889

	CGACAACCTGGTGCCGGGCTACCGCACCAACAACACGCT CTCGATCCGCCTGTGGAGCGCCAAGACCCCGCACGAGAT GGACCTGGCGGCGTTCAACGCCGGCGAGTACGGCCGCGC GTTCGAGAACAAGGTGCGCACGGAGACCATCACCTCCGT GCTCTACCCGAACGACCACCACTACAACGGCAAGGAGCT GCGGCTCAAGCAGCAGTTCCTCTTCGTCTCGGCCACGCTC CAGGACATCCTCAACCGCTTCAAGCGCCGCCACTTCGGC AAGAACCTCGAGCTCTA	

Appendix B: Bio-Bus Surveys

Bio-Bus Pre-Visit Survey for Students (Forensics)

Date of Module:
School:

Students,
Please use the following scale to indicate your level of agreement with the statements:

T= True, t=Somewhat True, N=Neutral, f = Somewhat False, F=False

I think that I could be a scientist.	**T**	**t**	**N**	**f**	**F**
No two people ever have the same fingerprints, even twins.	**T**	**t**	**N**	**f**	**F**
Science is interesting.	**T**	**t**	**N**	**f**	**F**
Blood type comes from genes from one parent only.	**T**	**t**	**N**	**f**	**F**
People who work in a science lab are cool.	**T**	**t**	**N**	**f**	**F**
I am interested in forensics.	**T**	**t**	**N**	**f**	**F**
DNA can be extracted from red blood cells.	**T**	**t**	**N**	**f**	**F**
I would like to learn more about forensics.	**T**	**t**	**N**	**f**	**F**
I feel like I can understand forensics.	**T**	**t**	**N**	**f**	**F**
I am usually good at science.	**T**	**t**	**N**	**f**	**F**
I learn better with hands-on activity than normal classes.	**T**	**t**	**N**	**f**	**F**
Saliva is useful for criminal investigations.	**T**	**t**	**N**	**f**	**F**
People who teach science are cool.	**T**	**t**	**N**	**f**	**F**
Science is one of my favorite subjects.	**T**	**t**	**N**	**f**	**F**

Please circle your gender:

Male / Female

In general, do you think that you could be a scientist? Why or why not?

Bio-Bus Post-Visit Survey for Students (Forensics)

Date of Module:
School:

Did you take a survey for the Bio-bus before today (a pre-visit survey)? (Circle one)

YES **NO**

Please use the following scale to indicate your level of agreement with the statements:

T= True, t=Somewhat True, N=Neutral, f = Somewhat False, F=False

I think that I could be a scientist.	**T**	**t**	**N**	**f**	**F**
No two people ever have the same fingerprints, even twins.	**T**	**t**	**N**	**f**	**F**
Science is interesting.	**T**	**t**	**N**	**f**	**F**
Blood type comes from genes from one parent only.	**T**	**t**	**N**	**f**	**F**
People who work in a science lab are cool.	**T**	**t**	**N**	**f**	**F**
I am interested in forensics.	**T**	**t**	**N**	**f**	**F**
DNA can be extracted from red blood cells.	**T**	**t**	**N**	**f**	**F**
I would like to learn more about forensics.	**T**	**t**	**N**	**f**	**F**
I feel like I can understand forensic science.	**T**	**t**	**N**	**f**	**F**
I am usually good at science.	**T**	**t**	**N**	**f**	**F**
I learn better when I do activities than when I just listen in class.	**T**	**t**	**N**	**f**	**F**
Saliva is useful for criminal investigations.	**T**	**t**	**N**	**f**	**F**
People who teach science are cool.	**T**	**t**	**N**	**f**	**F**
Science is one of my favorite subjects.	**T**	**t**	**N**	**f**	**F**

What was your favorite part of the Bio-Bus module today?

Please circle your gender:

Male / Female

In general, do you think you could be a scientist? Why or why not?