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About this Issue

Statement of Purpose

The Rhodes Journal of Biological Science is a student-edited publication, which recognizes the scientific achievements of Rhodes students. Volume XXIII marks the second year since journal was brought back into regular publication by Mark Stratton and Dr. David Kesler in 2006. Founded over twenty years ago as a scholarly forum for student research and scientific ideas, the journal aims to maintain and stimulate the tradition of independent study among Rhodes College students. We hope that in reading the journal, other students will be encouraged to pursue Scientific investigations and research.

Editorial Staff

Andrew Foss-Grant '10 (Senior Editor) is a biology and physics double major with a minor in mathematics from Dripping Springs, TX. He is a member of βββ Biological Honor Society, ΠΚΑ International Fraternity, and is the Treasurer for the Rhodes College chapter of the Society of Physics Students. Andrew has conducted research with former Rhodes professor Dr. Jim Armacost and will be doing further research this summer at the University of Michigan Biological Station. He enjoys tutoring for the physics department at Rhodes, and plans on continuing in the fields of Physics and Biology in either graduate school or medical school upon graduation.

Stephanie Juchs '08 (Senior Editor) is a biology major and environmental science minor from Bel Air, MD. She is a member of the βββ Biological Honor Society and has conducted research with Dr. Rosanna Cappellato and Dr. David Kesler of the Rhodes Biology department. Stephanie also enjoys volunteer work she has done with St. Jude, Habitat for Humanity, and with the Rhodes organization STAND where she mentors the children of Sudanese refugees. Stephanie is also the Service Learning Fellow for the SWEEP program where she teaches Cypress Middle School students about environmental issues. After graduation, she plans to attend graduate school at the University of Maryland to study urban ecosystems.

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Sarah Mercer '08 is a biology major from Robinson, TX. She is a member of the βββ Biological Honor Society, the ΗΣΦ Classical Honor Society, and Phi Beta Kappa. Sarah has conducted research with Dr. Terry Hill and Dr. Mary Miller of the Biology department and Dr. Darlene Loprete of the Rhodes Chemistry department. Sarah also enjoys interacting with puppies, eating out, and playing dominoes. After graduation, Sarah plans to attend Northwestern University to earn her PhD in molecular biology.

Student Contributors

Kim Green '09 is a biology major and psychology minor from Huntsville, AL. She is a member of the βββ Biological Honor Society, Mortar Board, ΧΑΣ National College Athlete Honor Society, ΧΩ Sorority, and Rhodes varsity swim team. Currently, Kim holds three individual swimming records at Rhodes College. In addition to academics and athletics, she also volunteers for Habitat for Humanity and at the Regional Medical Center. Kim was selected for the St. Jude Summer Plus program and conducted research from May 2006 – August 2007. Working with Dr. Lindquester, she studied the vIL-10 gene from the Epstein-Barr virus. After graduation, Kim plans on going to medical school and pursuing a career in medicine.

Frances Benoist '08 is a biology major from Fort Worth, TX. She is a member of the βββ Biological Honor Society and is conducting fungal cell wall research under the direction of Dr. Sara Gremillion, Dr. Terry Hill and Dr. Darlene Loprete. Currently, Frances is interning at LeBonheur Children's Medical Center where he is gaining valuable experience in the health field, which he plans to pursue. After graduation, she will spend a year continuing to gain medical experience while applying to medical schools.

Julia Goss '10 is a biology major from St. Louis, MO. She is a member of the BBB Biological Honor Society. Julia conducted her research at the St. Louis Zoo for the Animal Behavior Course at Washington University under the direction of Professor Robert Sussman of the Washington University anthropology department. Currently, Julia has an internship at the aquarium at the Memphis Zoo and is anticipating her trip for Coral Reef Ecology to Honduras. Julia is a member of the Rhodes College varsity track and field team and a member of the Kappa Delta sorority. After graduation, she plans to attend graduate school in Marine Biology to earn her Ph.D.

Tyler Cullender '08 is a biology major and an environmental science minor from Grand Prairie, TX. Tyler has conducted research under the direction of Dr. Keith Pecor and Dr. Mary Miller of the Rhodes Biology department and Dr. David Lodge of The University of Notre Dame. He is a member of the βββ Biological Honor Society and Rhodes Radio. Tyler enjoys playing guitar, cooking mediocre food, and composting. After graduation, he plans to attend Cornell University to earn his PhD in Microbiology.

Kristin Wheeler '09 is a biology major and Spanish minor from Jackson, TN. She is a member of βββ Biological Honor Society, Sigma Delta Pi Spanish Honor Society, Mortar Board, and Omicron Delta Kappa. In addition to academics and extracurricular activities, Kristin serves as the Student Associate for the Language Center. While pursuing undergraduate clinical experiences, she has participated in internships at Clínica Esperanza, Jackson Madison County General Hospital, and Planned Parenthood Greater Memphis Region. Kristin hopes to attend medical school in the fall of 2009 and plans to practice obstetrics and gynecology as her specialty.

Dustin Long '09 is a biology major and archaeology minor from Collierville, TN. He is a member of βββ Biological Honor Society and is the Student Associate for the archaeology department. During his undergraduate career he spent seven months in Tanzania, working as a diver within a tropical lake research project in Lake Tanganyika with the University of AZ and Bard College during the summer, and studying ecology and conservation with the School for International Training during the semester. In his time in Tanzania, he conducted ecological research in a mid-elevation montane rainforest. He has been accepted as a student fellow at the Woods Hole Oceanographic Institution for this summer. After graduation, he plans on pursuing graduate school.

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Functional analysis of Epstein-Barr virus viral interleukin 10 in a murine gammaherpesvirus model: vIL-10 contributes to splenomegaly but not to infectious titer or latency

Kim Green and Dr. Gary Lindquester
Department of Biology, Rhodes College

The Epstein-Barr virus (EBV) causes mononucleosis during the lytic infection of humans, but it is its ability to establish long-term latency that makes it a potential oncogenic pathogen. Some viruses, including EBV, express proteins that are homologous to those involved in regulating the immune system within the host. EBV encodes a homolog to the human interleukin 10 (IL-10) called vIL-10. In this study, the vIL-10 gene from EBV has been isolated and inserted into a murine gammaherpesvirus (MHV) via cotransfection. By infecting mice with the recombinant MHV, we are aiming to explicate the role of vIL-10 in viral infection, pathogenesis, and latency in vivo. Results indicate that increased levels of splenomegaly on day 14 p.i. are associated with the vIL-10 gene. However, there was little to no effect on latent virus titer with the addition of the vIL-10 gene.

Introduction

Herpesviruses are a leading cause of viral diseases amongst humans, second to only common cold viruses (Microbiology 2007). Herpesviruses possess several common characteristics. First, they all possess linear, double-stranded DNA. Additionally, compared to other viruses, herpesviruses have extremely large genomes. Within the viral genome, there are both essential and nonessential genes. In many herpesviruses, some of the non-essential genes likely function in evading the immune response of the host, thus enabling the virus to extend the lytic infection, as well as achieve latency. The most distinctive characteristic of herpesviruses, however, is their ability to establish a life-long infection in their hosts. Viral genome insertion into the host's nucleus allows for the virus to remain dormant and be replicated along with the host cell's DNA. In response to environmental cues, or after several cell replication cycles, the virus can be reactivated (Goldsby et al. 2003). The ability of herpesviruses to establish a life-long infection makes them potential oncogenic pathogens. For instance, Human Herpesvirus 8 is thought to cause Kaposi's sarcoma (NCI 2008).

The Epstein Barr Virus (EBV) belongs to the herpesvirus family and is the cause of infectious mononucleosis, a manifestation that occurs in 35-50% of cases. EBV establishes a latent infection in certain blood and throat cells, primarily B-lymphocytes, that remains for the rest of the life of the host. As much as 95% of the population in the US and other developed countries is infected with EBV (NCID 2007). In addition, EBV has been associated with a variety of cancers, including Burkitt's lymphoma, a high-grade, fast growing cancer that is rare in the US, but more common among children in central Africa (Cancerbackup 2007). EBV is also associated with nasopharyngeal carcinoma, a rare cancer that is more common among people of South Asian decent, arising in the epithelial of the nasophaynx (Mayo Clinic 2008). However, the role of EBV in tumorigenisis is not clear. The high rate of occurrence of EBV and its association with certain cancers make the study of this oncogenic pathogen very pertinent.

A central question in studying EBV (and all other herpesviruses) is how the virus manages to evade the host's immune system and establish latency. One possible answer is that EBV usurps the host's own immune control mechanisms. Some viruses, including EBV, code for proteins that are homologous to the immune regulating proteins produced by the host organism. The EBV viral interleukin 10 (vIL-10) protein, coded for by the vIL-10 gene, is a homologue to the human interleukin 10 (IL-10) protein. vIL-10 is 84% homologous to IL-10 and exhibits cytokine synthesis inhibitory activity on both mouse and human cells (Moore et al. 1990, Swaminathan et al. 1993, de Waal-Malefyt et al.1991, Niiro et al. 1992, Rousset et al. 1992). IL-10 unlike vIL-10 stimulates mast cells and induces proliferation of mature and immature thymocytes (Vieira et al. 1991, MacNeil et al. 1990). IL-10 and vIL-10 enhance B cell viability, whereas only IL-10 upregulates MHC II on B cells (Lockridge et al. 2000). Thus, though the viral and cellular homologs share many immunosuppressive activities, vIL-10 lacks IL-10's immunostimulatory functions. These differences have been attributed primarily to a single amino acid substitution (Ding et al. 2000). It would seem, then, that the vIL-10 protein provides EBV with a survival advantage – a way of suppressing the human immune system (Goldsby et al. 2003). However, this role of vIL-10 has yet to be experimentally confirmed. Due in part to the fact that previous studies have been hindered by a limited range of viral hosts, the role of the vIL-10 protein in viral pathogenesis is not yet clear.

In order to study the role of the vIL-10 protein, we chose to use the murine gammaherpesvirus (MHV) and the mouse model. Specifically, MHV-68 has been suggested as an animal model for human gammaherpesvirus infection, and, while it is notably different from EBV in some genetic and

functional characteristics, they share a pathogenic profile (Macrae et al. 2001). While MHV infection is generally inapparent in the natural host organism, *Clethrionomys glareolus* (the bank vole), infection by MHV-68 does cause pathogenic symptoms in the common house mouse and laboratory mouse, *Mus musculus* (Disease genome 2007). MHV-68 causes lymphocytosis in house mice similar to the EBV-induced mononucleosis in humans. Both viruses also achieve latency, primarily in B-cells (Macrae et al. 2001).

MHV-76 is a natural variant of MHV-68 and contains a 9.5-kilobase-pair (kbp) deletion of genes M1-M4. While this might seem to be a substantial deletion relative to the size of other viruses, in a herpesvirus, it is not. Also, this deletion is only of non-essential genes. MHV-76 infection in mice results in lower levels of splenomegaly, splenic latency, and lymphocytosis compared to MHV-68. In other words, MHV-76 is a less pathogenic viral strain (Macrae et al. 2001).

In this study, the mouse model, MHV-68, MHV-76, and recombinant MHV-76 containing the vIL-10 gene from EBV were used in an attempt to characterize the role of the vIL-10 protein in lytic infection and in establishing latency. It was hypothesized that introducing the EBV IL-10 gene into MHV-76 will restore the high levels of lymphocytosis, due to the high proliferation rate of Th1 cells, splenomegally, and viral latency associated with MHV-68. This hypothesis does not imply that the 9.5 kbp deleted region in MHV-76 is equivalent to the vIL-10 gene, but that since the vIL-10 gene is known to hinder the certain immune response, it might increase the likelihood of the virus establishing latency.

Materials and Methods

Creating plasmids used in generating recombinant MHV-76

The strategy for creating recombinant MHV-76 is illustrated in Fig. 1 and Fig. 2. Briefly, Pgp150 (a MHV late gene promoter) and Ppgk (a murine housekeeping promoter) were isolated by PCR from the MHV68 gp150 gene and the mouse 3-phosphoglycerate kinase (pgk) gene, respectively. Each promoter was then inserted into the pGL3-Basic vector (Promega), and tested for ability to drive luciferase expression. The luciferase gene was replaced by the EBV vIL-10 gene, and vIL-10 expression was verified by an IL-10 Enzyme-Linked ImmunoSorbent Assay (ELISA) which confirmed the presence of the vIL-10 protein in cells. The promoter-vIL-10 fragments were then inserted in either orientation into a targeting plasmid such that they were bounded by a region containing a nearly complete copy of the MHV terminal repeat (TR) and a 3 kb fragment from the prototype left end of MHV76 adjacent to the terminal repeat (LHE).

Generating/Analyzing recombinant MHV-76

Generation of recombinant MHV-76 is outlined in Fig. 3. The recombinant MHV-76 virus was generated through co-transfection of NIH 3T3 cells (mouse embryonic fibroblast cells). *PmeI* was used to liberate fragments containing the terminal repeat, promoter, vIL-10 and the LHE regions from the plasmids. These fragments along with MHV76 viral DNA were transfected into NIH-3T3 cells using FuGENE (Roche) according to the manufacturer's recommendations. Cultures were incubated at 37ºC with 5% CO_2 for 5-6 days until plaques formed. Cells from co-transfection were harvested and frozen/thawed three consecutive times using dry ice bath and 37ºC water bath in order to lyse the cells and release the virus. 2-5 µL of the culture supernatant were added to the first column of wells of a previously seeded (1×10^4 cells/well in 200 µL of DMEM) 96-well plate. A 2-fold serial dilution was created by transferring 100 µl of media from the first column of wells on the plate to the second column, and repeating this step across the plate. The plate was incubated at 37ºC with 5% CO_2 for 5-6 days until plaques formed. Wells containing 1-2 plaques were harvested. The viral DNA from these cells was purified and screened. PCR on the resulting viral DNA was done using vIL-10 primers, as well as primers specific to the gp150 promoter within MHV-76. Results were considered "positive" if PCR products from both the vIL-10 and gp150 primers produced bands in the gel. Left and right orientation of the insertion was determined by different restriction enzyme analysis.

Generating/Analyzing revertant MHV-76

Generation of revertant MHV-76 is outlined in Fig. 4. A revertant virus, which served as an added negative control, was generated by essentially reversing of the previously described procedure using recombinant virus containing the Pgp150-vIL10 insert in the leftward orientation and the fragment containing the TR and LHE regions of MHV-76. PCR screening was done using vIL-10 primers and the primers specific to the gp150 promoter. Results were considered "positive" if bands in the gel were only seen in the PCR product that used the gp150 primer. The lack of a band with use of the vIL-10 primers indicated that the vIL-10 gene was successfully removed.

Growth rate curve

A single-step growth rate curve was generated for MHV-68, MHV-76, rMHV76.Pgp150.vIL10L, and rMHV76.Pgp150.vIL10L.Rev in order to ensure that any differences in viral pathogenesis *in vivo* would be due to the differences in the v-IL10 expression, and not the difference in rate of growth. 10 wells (of 6 well plates previously seeded with 3×10^5 cells/well) were infected with 5 multiples of infection (moi) per cell for each virus in a total of 2.2 ml of DMEM (a total of 20 wells, 10 for rMHV76, and 10 for MHV76). Plates were incubated for 1 hour at 37ºC with 5% CO_2. Media was aspirated, and wells were washed twice with PBS. 5 mls of fresh media were added to each well, marking time 0. Samples were taken at 0, 4, 8, 12, 16, 20, 24, 36, 48, and 72 hours. Samples were frozen/thawed three times to release the virus from the cells, and titrations in duplicate of each sample were done to determine the amount of virus present.

Infection of mice and collection of tissue

3-5 week-old BALB/c mice were divided into 5 groups: MHV-68, MHV-76, MHV76.Ppgk.vIL10R, MHV76.Pgp150.vIL10L, and MHV76.Pgp150.vIL10Lrev infected. Mice were infected intranasally under anesthesia (Isoflurane). Each mouse was infected with 2×10^5 plaque

forming units (pfu) of appropriate virus in 40 µl of PBS. Mice from each group were sacrificed by CO_2 asphyxiation at day 6, 11, and 15 post infection (p.i.). Spleens were harvested on days 10, 14, and 21 p.i. Spleens from the same infection group were combined in the same 15 ml centrifuge tube containing 5 ml of PBS and kept on ice until splenocyte assays were done.

Splenocyte assays

Isolation and counting of primary splenocytes were performed as soon as possible after completion of harvest. Spleens were placed on a 70 µm strainer set loosely on top of a 50 ml centrifuge tube, and then disrupted with the blunt end of the plunger from a 5 ml syringe. Cells were washed through using 10 ml of PBS to ensure collection of most of the cells. The disrupting/washing was repeated, and cells were pelleted at 4ºC for 10 minutes at 1500 rpm. Cells were resuspended and mixed for 1 minute in red cell lysis buffer (Sigma) at 1 ml per spleen. After sitting for one minute, the cell suspension was filtered into a clean tube and washed with 20 ml PBS. Cells were then pelleted, resuspended in 5 ml DMEM, and counted using a hemocytometer.

Latent Virus Titer

In order to determine the total amount of latent virus present a limiting dilution reactivation assay on the splenocytes was performed by adding 3.2 x 10^6 cells in 3.2 ml of DMEM to a reservoir (kept on ice). 100 ul of dilution was added to each well in the first two columns of a 96 well plate (previously seeded with 5 x 10^3 MEF cells). 1.6 ml of media was added to the reservoir, and 100 µl of dilution was added to each well of the next two columns. This step was repeated until 12 dilutions were plated. Dilutions were done in duplicate such that there were 16 wells of each dilution. Wells were scored for cytopathic effect (CPE) on days 14 and 21 p.i.

Lytic Virus Titer

In order to determine background of lytic virus in the splenocytes, splenocytes (3.2 x 10^6 cells in 1 ml DMEM) were disrupted in a beadbeater and titrated by dilution on NIH 3T3 cells in order to determine the amount of lytic virus present in the cells. Plates were read 5-6 days after infection.

Results

Generation of recombinant and revertant MHV-76

MHV-76 is a natural mutant of MHV-68, containing a 9.5 kbp deletion of genes M1-M4, resulting in lower levels of splenomegaly, splenic latency, and lymphocytosis upon infection compared to MHV-68. The EBV vIL-10 gene was successfully inserted into a plasmid containing either Pgp150 or Ppgk (Fig.1). Promotor/vIL-10 fragments were then inserted in either orientation into a targeting plasmid containing a nearly complete copy of the TR and the LHE of MHV76 (Fig.2).

Once plasmids containing the MHV-76 TR, LHE, and the EBV vIL-10 gene with promoter were created, recombinant MHV-76 was generated by co-transfection (Fig. 3). Results (not shown) of sequencing and restriction enzyme digestion illustrated the successful creation of several recombinant MHV-76 stains including MHV76.Pgp150.vIL10L (virus containing the gp150 promotor and the vIL-10 gene in the leftward orientation), and MHV76.Ppgk.vIL10R (virus containing the pgk promoter and the vIL-10 gene in the rightward orientation). The revertant virus, MHV76.Pgp150.vIL10L.Rev, was made by removing the vIL-10 gene from MHV76.Pgp150.vIL10L (Fig. 4).

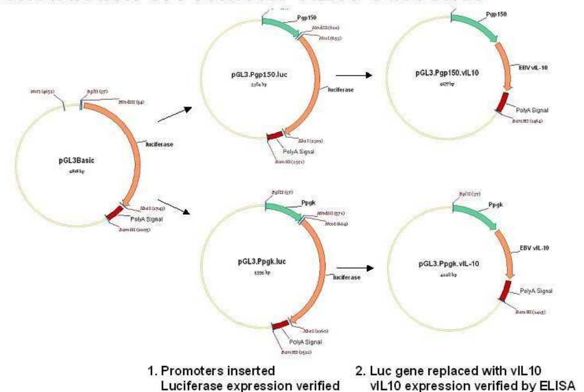

Figure 1: Promoters Pgp150 and P.pgk were inserted into the pGL3-Basic vector (Promega), and tested for ability to drive luciferase expression. The luciferase gene was replaced by the EBV vIL-10 gene, and vIL-10 expression was verified by ELISA.

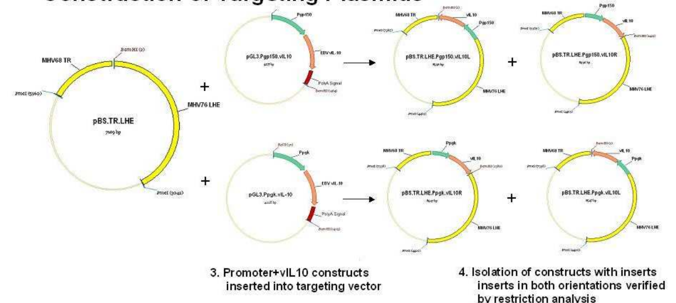

Figure 2: Promoter/vIL-10 fragments were inserted in either orientation into a targeting plasmid. Insertions were bounded by a region with a nearly complete copy of the MHV TR and a 3 kb fragment from the LHE.

Growth Curve

Once the recombinant viruses were made, a single-step growth curve of MHV-68, MHV-76, MHV76.Pgp150.vIL10L, and MHV76.Pgp150.vIL10L.Rev was created. Results show that viral growth rates *in vitro* are essentially the same (Fig. 5).

Splenocyte Counts

Results of splenocyte counts indicate a significant increase in splenocyte number from vIL10-containing viruses on Day 14 p.i. compared to MHV-76 or the revertant control but not as high as MHV-68 (Fig. 6). At 14 days p.i., splenomegaly occurred to a greater extent in mice infected with the recombinant MHV-76 viruses containing the vIL-10 gene than in those infected with wild-type MHV-76.

Latent and lytic virus titrations

Once the splenocyte count was obtained, limiting dilution assays (with both live cells and disrupted cells) were done to determine the amount of virus, both lytic and latent, within the cells. Results indicate no difference between MHV76 and recombinants with or without vIL10 (Fig. 7). Results also show that all of the viruses achieve latency in the spleen, but MHV-68 does so at a much higher level, consistent with published studies comparing MHV-68 and MHV-76 (Macrae et al. 2001, Townsley et al. 2004).

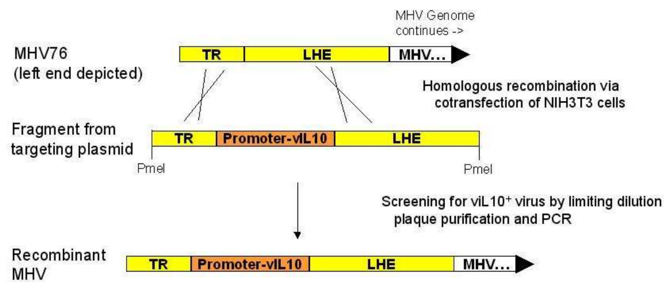

Figure 3: Fragments from the targeting plasmid were inserted into the wild-type MHV-76 through homologous recombination via co-transfection of NIH3T3 cells.

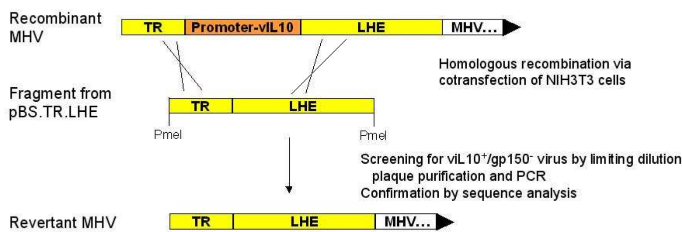

Figure 4: Revertant MHV-76 was made by essentially reversing procedure used to make the recombinant MHV-76 containing the promoter and the vIL-10 gene.

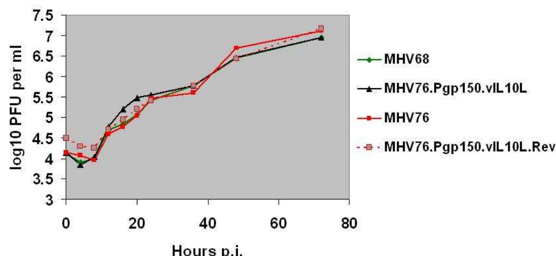

Figure 5: Viruses have equivalent *in vitro* growth properties. Samples of cells infected at 5 m.o.i per cell were collected at 0, 4, 8, 12, 16, 20, 24, 36, 48,and 72 hours. Samples were titrated in duplicate to determine the number of pfu per ml of sample.

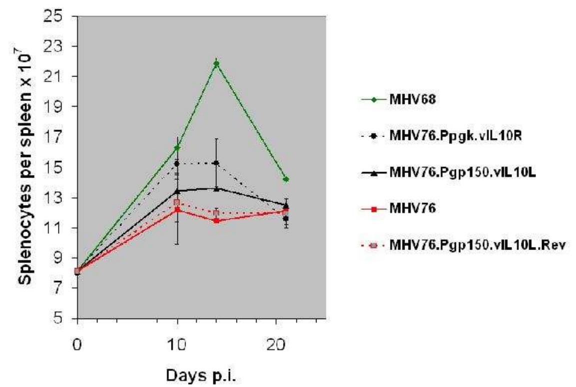

Figure 6: Data represent two independent experiments with five mice per group. Spleens were harvested on days 10, 14, and 21 p.i. Spleens of five mice were pooled and disrupted. Splenocytes were counted. Splenocyte count data for Day 0 represent the average of five spleens from uninfected mice. Bars represent standard error of the mean.

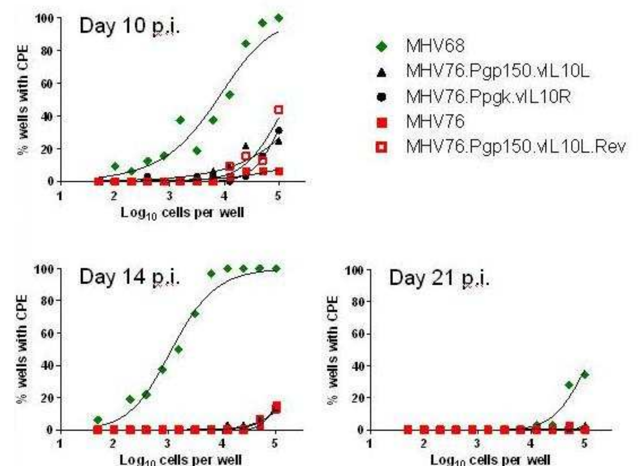

Figure 7: In this limiting dilution analysis (Ding et al. 2000) spleenocytes were plated atop MEF cells in dilutions across 96-well plates as indicated on the X-axes with 16 duplicate wells per dilution. Wells were scored for CPE on days 14 and 21. Curve fit lines represent results of nonlinear regression analysis. Data points represent the mean of two independent experiments with spleens pooled from five mice per experiment.

Discussion

In this study, the role of the vIL-10 gene in establishing viral infection and latency was addressed. Recombinant herpesviruses consisting of MHV76 with an EBV vIL-10 insertion driven by the MHV gp150 or pgk promoter were generated. The viral pathology of rMHV76.Pgp150.vIL10L and rMHV76.Ppgk.vIL10R was compared to that of MHV-68 and MHV-76. It was predicted that the pathogenic profile of the recombinant MHV-76 containing the vIL-10 gene would more closely resemble that

of MHV-68 rather than MHV-76. Specifically, it was predicted that introducing the EBV IL-10 gene into MHV-76 would restore the high levels of lymphocytocis, due to the high proliferation rate of Th1 cells, splenomegaly, and viral latency associated with MHV-68. Infection of BALB/c mice, however, did not support this hypothesis. Results indicated that vIL-10 leads to a moderate expansion of splenocyte populations at day 14 p.i. but does not significantly affect the quantity of virus able to reactivate from latently infected spleenocytes.

Experiments are underway to determine if viral lung titers at days 5, 10 and 14 p.i. are significantly affected by the presence of vIL-10. In addition, the quantity of latent virus as determined by PCR analysis is being addressed. Future experiments will assess the expression of vIL-10 in infected mice and changes in host cytokine expression upon infection.

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Fungal Vaccines: The Search for the Antigen

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Recent research on possible fungal vaccines has shown very important and helpful developments in the mechanism of vaccine protection. The search for a Coccidioides spp. vaccine began in the 1950s, but the failure of the formalin-killed spherule vaccine against this fungus suggests possible complications in creating a vaccine. The identification of new immunogenic fungal antigens is one important development because it is most probable that isolation of known antigens from the nonantigenic spherule material can increase the immune response. In addition to finding the best fungal antigen, current research shows that a combination of multiple antigens into one fungal vaccine could significantly offer more protection than each antigen alone. While the necessity of fungal vaccines is a controversy still evident in many research institutions, current research for fungal vaccines is, nonetheless, present and making breakthroughs in the creation of a fungal vaccine.

Introduction

The development of fungal vaccines is a relatively recent approach to preventing fungal infections, but a successful fungal vaccine for humans has not yet been created. However, there are many new and promising possibilities for a potential fungal vaccine. Most promising are fungal subunit vaccines which isolate a specific immunogenic antigen to elicit an immune response and provide prolonged protection from that specific pathogen (Calderone *et al.* 2002). While research to find a fungal vaccine is ongoing, there is still a controversy as to whether a vaccine is even necessary. While one might argue that any life-threatening disease is worth trying to prevent, there are definitely arguments against fungal vaccines. Heitman *et al.* (2006) explain that the main argument against fungal vaccines is the lack of data pertaining to fungal diseases. There is very little knowledge of how prevalent fungal diseases are because they do not have to be reported to health boards, and, therefore, an estimated profit from fungal vaccines cannot be calculated (Heitman *et al.* 2006). In the study of fungal diseases, it is important to note that fungal vaccines must be a marketable product and pharmaceutical companies must have an incentive to produce them. Heitman *et al.* (2006) go on to explain that when the cost-benefit for a vaccine against *Coccidioidis immitis* was determined, the estimated gain would be about $3,000,000 each year. Despite this discovery, the argument against fungal vaccines continues. Nonetheless, there are many scientists at different research institutions that have chosen to look past the controversy of a questionable profit and search for a fungal vaccine.

Immunization by a vaccine can be acquired passively or actively, depending on the type of vaccine. An active vaccine elicits an immune response in the injected patient, and a passive vaccine provides protection by injection of certain immune response products such as antibodies or lymphocytes (Heitman *et al.* 2006). An active vaccine is often delivered before an infection, and it causes an immune response which creates certain antibodies and memory cells which are antigen-specific for that pathogen and remain in the body for future protection (Heitman *et al.* 2006). A passive vaccine only offers short-term protection and is often given to immunocompromised patients to aid in controlling a chronic infection (Heitman *et al.* 2006). The three main types of active vaccines are live attenuated, inactivated, or subunit (Enquist *et al.* 2000). Of these types of vaccines, the subunit vaccine is the most predominantly researched type of fungal vaccine. This is due to the fact that an isolated immunogenic antigen has a better chance of eliciting an immune response, and it is easier to isolate out and find the best vaccine candidates by creating subunit vaccines and testing the efficacy of different antigens one at a time (Calderone and Cihlar 2002).

In the search for a fungal vaccine, the key to eliciting a protective immune response is to find the fungal antigen, or immunogen. Therefore, most research focuses on finding these fungal immunogens and test their immunogenicity on murine and other models. There are also certain fungi which are better candidates for creating a fungal vaccine against, and these are the ones in which known fungal immunogens are being studied to elicit a protective immune response (Heitman *et al.* 2006). The best fungal candidates for a vaccine are the following: *Aspergillus* spp., *B. dermatitidis, Candida* spp., *Coccidioides* spp., *C. neoformans, H. capsulatum, P. brasiliensis,* and *Pneumocystis* (Heitman *et al.* 2006). Of these candidates, *Coccidioides* spp. is a major fungal target for the creation of a protective vaccine. This is due to the fact that coccidioidomycosis is endemic to a particular region, and healthy individuals who have overcome an infection are often protected from a subsequent infection (Esser and Bennett 2004). The search for a *Coccidioides* spp. vaccine began in the 1950s, and a formalin-killed spherule vaccine was even experimented on 3,000 people in the 1980s (Esser and Bennett 2004). Unfortunately, the FKS human vaccine did not show any protection against coccidioidomycosis. This failure could be attributed to too much nonanitgenic material in the cell wall, which reduced the vaccine's protection. Another theory for its failure was the fact that the FKS vaccine, in too high of a concentration, was harmful to the patient receiving the vaccine (Esser and Bennett 2004). Therefore, the failure of this vaccine made it evident that a specific fungal antigen must be isolated to create a successful vaccine. An isolated antigen,

without the surrounding nonimmunogenic material, could elicit a stronger immune response without causing as much harm to the patient.

Discussion

Since the FKS vaccine of the 1980s, many scientists have researched the different fungal immunogens located on the spherule outer wall of *Coccidioides* spp. A review article published by Zimmermann *et al.* (1998) describes the protective ability of a multicomponent fraction isolated from the spherule wall of *C. immitis*. The soluble aqueous fraction, termed 27K vaccine, was isolated from the FKS vaccine by mechanical disruption, the protective ability of 27K was tested in comparison to the FKS vaccine. In the experiment, seven mice were injected with 27K plus an alum adjuvant, 27K alone, or the alum adjuvant alone (Zimmermann *et al.* 1998). The mice were then injected with three different amounts of arthroconidia, intranasally and intravenously, four weeks after the third dose of their specific injection (Zimmermann *et al.* 1998). Figure 1, from Zimmermann *et al.* (1998), shows that there is a significant difference of survival between mice injected with 27K plus alum compared to 27K alone when challenged intravenously with 500 and 5,000 arthroconidia and intranasally with 5,000 and 15,000 arthroconidia. There is a significant difference in mouse survival between 27K plus alum compared to alum alone when challenged intravenously with 500 and 5,000 arthroconidia and intranasally with 5,000 arthroconidia (fig. 1). Figure 1 also shows that the 27K vaccine, when injected with an alum adjuvant, is almost as protective as the FKS vaccine it was prepared from (Zimmermann *et al.* 1998).

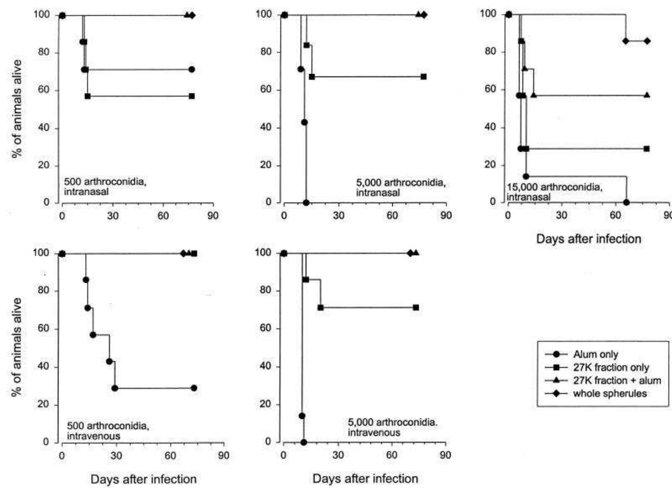

Figure 1: Mouse survival, when injected with 27K + alum is significantly different from both 27K alone and alum alone in most of the different challenges with arthroconidia. Also, the 27K + alum is almost as protective as the FKS vaccine (whole spherules). The intranasal infection with 15,000 arthroconidia shows less protection from 27K + alum than FKS. (Zimmerman *et al.*, 1998)

Zimmermann *et al.* (1998) go on to describe the different attempts to separate the components of the 27K vaccine. Researchers attempted to fractionate 27K vaccine by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the Coomassie blue-stained gel did not show separated bands, but only a long smudge across the gel (fig. 2). Zimmermann *et al.* (1998) were then able to successfully fractionate the 27K vaccine by isoelectric focusing (IEF) showing separate bands of on Coomasie blue-stained IEF gel (fig. 3). When figure 2 is compared to figure 3, it is evident that figure 3 shows the separated components of 27K vaccine. It is important to realize that 27K is made up of different components and not an isolation of one antigenic molecule of the spherule wall. The different components of 27K could have different protective abilities against *C. immitis*, and further knowledge of the components could help in increasing anitgenicity of the vaccine.

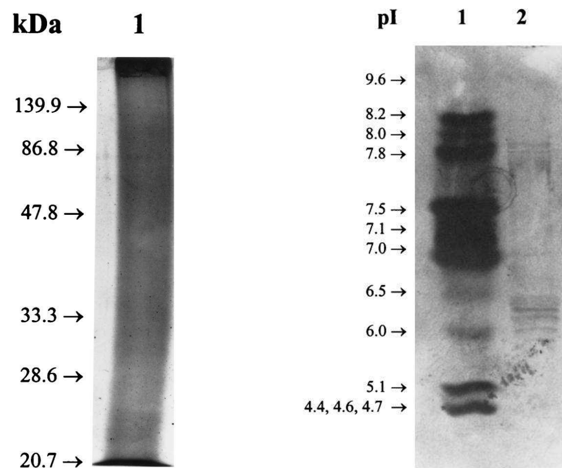

Figure 2: 27K was unsuccessfully fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis because the Coomasie blue-stained gel did not show separate bands. (Zimmerman *et al.*, 1998)

Figure 3: 27K was successfully fractionated because the Coomasie blue-stained IEG gel shows separate bands, especially when compared to fig. 2. (Zimmerman *et al.*, 1998)

While 27K was able to show similar protection to FKS, a successful vaccine must surpass the protective ability of FKS. Zimmermann *et al.* (1998) make the point that isolation of specific immunogenic components of the 27K vaccine could be crucial in increasing the vaccine's protection against *C. immitis*. Another possible improvement to 27K vaccine's efficacy could be the addition of a better adjuvant because adjuvants may enhance the cell-mediated immunity, a branch of the immune system critical in fighting fungal infections (Zimmermann *et al.* 1998). It is clear in figure 1 that the 27K vaccine, which was injected along with the alum

adjuvant, shows the best protection when compared to the FKS vaccine, but a new and improved adjuvant would be helpful in increasing the immunogenicity of 27K (Zimmermann *et al.* 1998).

Research for a fungal vaccine against *Coccidioides* spp. makes it clear that the search for the best antigen may be an integral part in finding a successful fungal vaccine. Specific immunogenic antigens, coupled with an optimal adjuvant, could be the key to providing protection against life threatening fungal diseases. One study by Kirkland *et al.* (1998) examined the immunogenic protection of a specific component of the *C. immitis* spherule wall, proline-rich antigen (PRA). PRA has been known to elicit an immune response in both the humoral and cell-mediated branches of the immune system, and Kirkland *et al.* (1998) specifically studied PRA's ability to elicit a T cell response and provide protection for mice infected with *C. immitis*. Mice were immunized with the purified protein (rPRA), and T cell proliferation was measured ten days later (Kirkland *et al.* 1998). Results from this study (fig. 4) show that rPRA caused a large proliferation of T cells after being immunized with rPRA, but immunization with rPRA did not cause T cell proliferation in response to mycelial filtrate-plus-lysate fraction (mycelial F+L) (Kirkland *et al.* 1998). Kirkland *et al.* (1998) explain that these results show that rPRA is able to immunize mice against rPRA, but rPRA does not provide T cell protection against mycelial F+L. This is probably due to the fact that PRA is not expressed in *C. immitis* mycelia, and, therefore, there is no antigenic T cell response to rPRA (Kirkland *et al.* 1998).

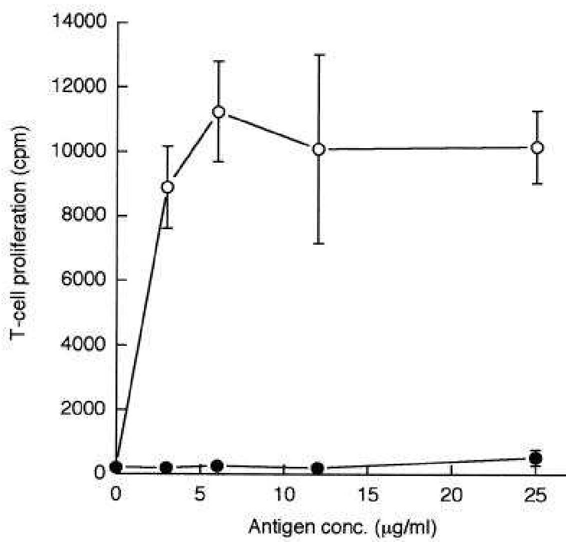

Figure 4: PRA is able to elicit T cell proliferation, but there is very little T cell response when the PRA-immune mice are exposed to mycelia F+L. PRA is probably not expressed in mycelia. (Kirkland *et al.*, 1998)

Kirkland *et al*. (1998) also shows that rPRA is able to provide protection for mice infected with *C. immitis.* Mice immunized with rPRA had significantly less amounts of organisms in their lungs and spleen when compared to nonimmunized mice (fig. 5). As figure 5 shows, the median CFU in rPRA-immune mice was 3.3 in the lungs and 3.37 in the spleen, and, showing much less protection, the median CFU in nonimmune mice was 6 in both the lungs and spleen (Kirkland *et al.*, 1998). Kirkland *et al.* (1998) also used FKS to immunize mice, and the results showed no viable organisms in the lungs and spleen. This is probably due to the fact that FKS has an increased antigenicity because it is a whole spherule, rather than an isolated spherule wall component. This experiment also shows the need to develop new adjuvants which can be added to certain antigens to elicit a greater immune response. While PRA was injected along with the incomplete Freund's adjuvant (IFA), it appears that perhaps a stronger adjuvant could aid in PRA's ability to elicit an immune response (Kirkland *et al*., 1998). Kirkland *et al*. (1998) show that PRA is able to stimulate some T cell proliferation and provide more protection than the control, but increased antigenicity must be developed for successful protection.

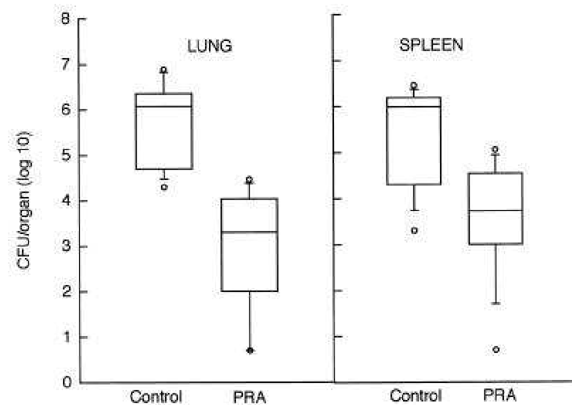

Figure 5: PRA-immune mice show protection against *C. immitis* infection when compared to nonimmune mice. The CFU values are significantly different in both the lungs and spleen. (Kirkland *et al.*, 1998)

The results of this research show that a specific spherule wall antigen was successfully isolated, and the isolated protein elicits a T cell response in mice which provides some protection from a subsequent infection with *C. immitis*. This information enhances and expands upon the identification of 27K lysate which is also able to provide protection against *C. immitis*. While 27K is made up of many unknown components of the spherule wall, PRA is one specific antigen isolated from the spherule wall. This development from an undifferentiated lysate to the isolation and description of a specific antigen is steady development in the search for a vaccine against *C. immitis.* Finding an antigen is a long process which begins with a general search for antigens and leads into more specific possibilities for creating a fungal vaccine.

In the search for a fungal antigen to create a successful fungal vaccine, it is important to learn the mechanisms by which the antigen provides protection against the pathogen. Dionne *et al.* (2006) studied the effects of *C. posadasii* spherules on human dendritic cells (DC),

specialized antigen presenting cells often used by the body to fight off fungi and other pathogens. Figure 6 shows a specific experiment in which immature DC were incubated with FITC-labeled spherules, then were fluorescently labeled with DC-specific antibodies, and finally fixed and permeabilized to observe the specific antibody markers inside and outside the DC (Dionne *et al.*, 2006). Immature DC which were incubated for 24 hours with FITC-labeled spherules show that DC (red) often ingested 1-6 spherules (green) in this time period (fig. 6). Furthermore, immature DC which were incubated for 72 hours with FITC-labeled spherules show that DC were able to digest and present spherule antigens (yellow and orange) on the surface of the DC (fig. 6). Dionne *et al.* (2006) then saturated DC with spherules, and over 90% of the DC took up and ingested the spherules. DC are clearly an important line of defense when fighting off *C. posadasii.*

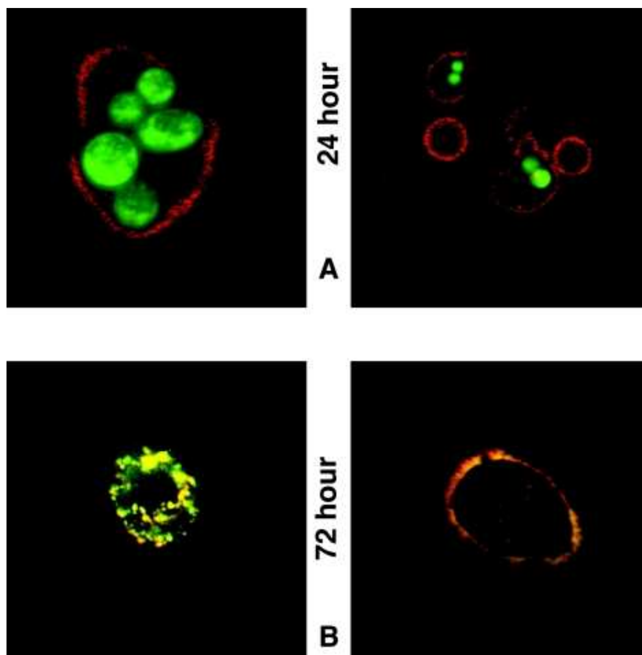

Figure 6: DC (red) phagocytize and ingest FITC-labeled *C. posadasii* spherules (green) after 24 hours. DC digest and display antigens on the outside of DC (yellow/orange) after 72 hours. (Dionne *et al.*, 2006)

Dionne *et al.* (2006) were also interested in the morphological maturation of DC, when cultured with *C. posadasii* spherules. Figure 7 shows the difference between immature DC, DC cultured with TNF-α and PGE_2 (a combination known to activate DC), and DC cultured with spherules (Dionne *et al.*, 2006). The mature DC (cultured with TNF-α and PGE_2) look very similar to DC cultured with spherules, indicating that spherules are able to cause the morphological maturation of DC (fig. 7). Images B and C appear to have similar phenotypes which consist of long dendritic processes, typical of mature DC, while A lacks these processes (fig. 7). Therefore, the similarity between mature DC in image B and immature DC cultured with spherules in C shows the ability of spherules to induce DC maturation. Fungal spherules apparently have a significant effect on DC maturation, a mechanism critical to finding the best fungal antigen to be used in a vaccine. The antigen should probably, similar to whole spherules, be able to induce DC maturation into functioning antigen presenting cells.

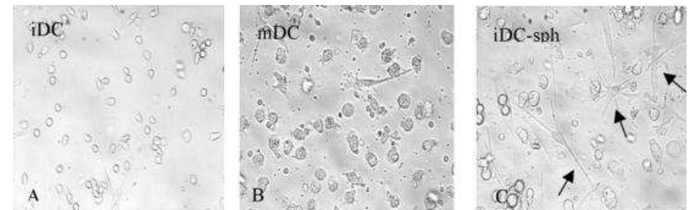

Figure 7: Morphology of (A) immature DC compared to (B) mature DC cultured with TNF-α and PGE_2 and (C) DC cultured with *C. posadasii* spherules. Arrows point to mature DC with long cell processes seen in both B and C. (Dionne *et al.*, 2006)

Dionne *et al.* (2006) show another interesting finding in which DC are able to present antigens and activate nonimmune PBMC (peripheral blood mononuclear cells). Figure 8 shows that DC cultured with spherules are able to induce proliferation of nonimmune PBMC. This experiment even shows that there is a significant difference between PBMC activation by DC cultured with spherules and any other application, which consist of immature DC, mature DC, T27K, spherules, and a control PBMC only (fig. 8). This data is interesting and helpful in the search for the best fungal antigen because it shows that T27K, the same vaccine studied by Zimmermann *et al.* (1998), is not able to activate nonimmune PBMC in the absence of DC (Dionne *et al.*, 2006). DC must be available to present antigens to lymphocytes and activate the cell-mediated branch of the immune system, previously found to be crucial in fighting fungal infections (Zimmermann *et al.*, 1998).

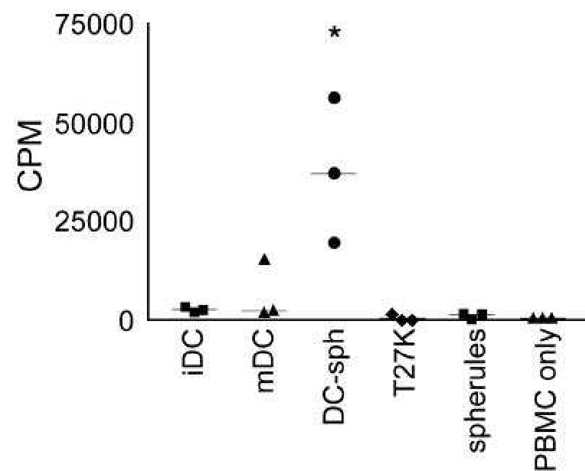

Figure 8: DC cultured with spherules are able to activate PBMC, while all other applications are not. There is a significant difference in the proliferation of lymphocytes in DC-sph compared to immature DC, mature DC, T27K, spherules, and PBMC alone. (Dionne *et al.*, 2006)

Dionne *et al*. (2006) continue with their research on DC by establishing a possible antigen to be used in a vaccine against *C. posadasii*. Immature DC were incubated with mannan, which acts to inhibit mannose receptors on DC, spherule binding was reduced by about 50% (Dionne *et al.*, 2006). This data provides direction in the search for a fungal antigen because mannose receptors are involved in DC binding to spherules, and antigens such as spherule wall mannoproteins could be studied as possible components in a fungal vaccine. The research by Dionne *et al*. (2006) is very helpful in the search for a fungal vaccine against *Coccidioides* spp. because it shows the importance of dendritic cells in the activation of the immune system. Further research can use this information to find a specific antigen, or antigens, that cause the maturation of DC which are then able to phagocytize and digest spherules. One of the most important functions of DC is the presentation of antigens to lymphocytes in order to activate the adaptive immune response, and the research by Dionne *et al.* (2006) clearly portrays the importance and possible target that DC could be in the creation of a successful vaccine.

Tarcha *et al*. (2006) advance the search for a fungal antigen even further by identifying a set of fungal cell wall proteins which contain various MHC II binding epitopes, which will bind specifically to antigen presenting cells in the immune system. This is an important discovery because it shows the use of multiple antigens combined in a vaccine to provide protection for mice. Mice immunized with the multicomponent vaccine, consisting of T cell reactive antigens, are protected from a subsequent infection with *C. posadasii* (Tarcha *et al.*, 2006). In order to ensure that the proteins isolated do, in fact, contain MHC II binding sites, Tarcha *et al.* (2006) used the ProPred algorithm to predict the presence of human MHC II-specific epitope sequences (fig. 9). Expression of the genes PEP1, PLB, and AMN1 were found to be responsible for the T cell reactivity of the antigenic proteins Pep1, Plb, and Amn1 (fig. 9), and the expression levels of these three genes were then studied in the different parasitic phases of *C. posadasii* (Tarcha *et al.*, 2006). PLB and AMN1 expression is the highest during the early phase of spherule development, before segmentation, while PEP1 has a stable expression throughout the spherule cycle (fig. 10) Expression of all three genes is relatively low during the segmentation stage, and PLB expression is higher than AMN1 and PEP1 in the last phase of endosporulation (fig. 10). These findings show that these three different genes encode for different proteins during different parasitic phases of the spherule, and, therefore, are potential antigens to be used in a vaccine against *C. posadasii* (Tarcha *et al.*, 2006).

TABLE 2.

Deduced proteins of *C. posadasii* selected on the basis of predicted cell wall association and analyzed by the ProPred algorithm for presence of epitopes which bind to human MHC class II-restricted molecules

Gel spot no.	Selected seroreactive protein[a]	Predicted molecular mass (kDa)	No. of predicted promiscuous T-cell-reactive epitopes[b]	Ratio of no. of predicted promiscuous epitopes to molecular mass (kDa) of deduced protein
2	Aspartyl protease (Pep1)	43.5	5	0.115
14	Phospholipase B (Plb)	68.6	7	0.107
3	Alpha-mannosidase (Amn1)	56.9	6	0.105
1	Chitinase 1 (Cts1)	47.4	5	0.104
15	Beta-glucosidase 4 (Bgl4)	32.9	3	0.091
8	Exo-1,3-beta-D-glucanase	93.1	8	0.085
5, 6	Endo-1,3-beta-glucanase	96.3	8	0.083
7	Carboxypeptidase Y	60.3	5	0.083
12	Beta-glucosidase 2 (Bgl2)	92.8	5	0.054
11	Beta-glucosidase 5 (Bgl5)	56.7	3	0.053
9	Protein disulfide isomerase	57.3	2	0.035
13	Spherule outer wall glycoprotein (SOWgp)	46.3	1	0.021
4	Metalloprotease 1 (Mep1)	29.7	0	0
10	Proline/threonine-rich protein	29.0	0	0

[a]Protein identity based on homology to reported fungal proteins determined by BLAST searches (1).

[b]Based on sequence analysis with the ProPred algorithm. A promiscuous epitope is predicted to be presented by at least 80% of the 51 HLA alleles tested in the ProPred algorithm (37).

Figure 9: The antigenic proteins Pep1, Plb, and Amn1 (corresponding to gel spot numbers 2, 14, and 3) are found to contain multiple T cell reactive epitopes (MHC II binding sites). This data was found by using the ProPred algorithm to analyze sequences. (Tarcha *et al.*, 2006)

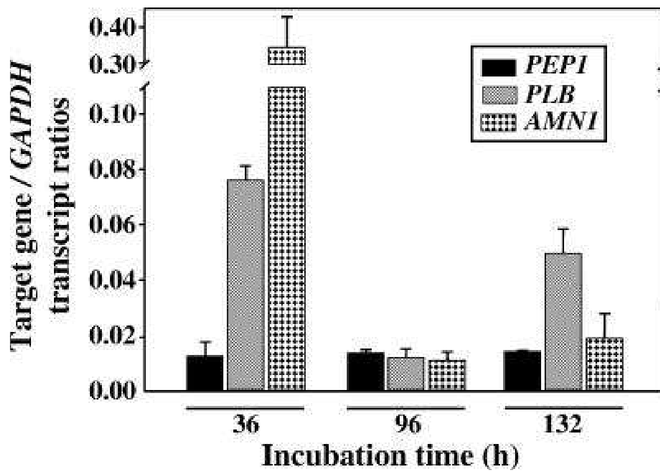

Figure 10: Expression of PEP1, PLB, and AMN1 in the *C. posadasii* parasitic cycle. PLB and AMN1 have the highest expression in the presegmentation phase (36 h), all are relatively low during segmentation (96 h), and PLB is expressed the most during endosporulation (132 h). (Tarcha *et al.*, 2006)

The purified recombinant proteins rPep1, rPlb, and rAmn1 were then used to immunize mice separately and together before being infected with a lethal amount of *C. posadasii* (Tarcha *et al.*, 2006). Both the percentage of mice which survived the infection and the amount of CFU in the lungs revealed that the combination of all three recombinant proteins provided more protection than each recombinant protein alone (fig. 11). While each single protein provided significantly more protection than the PBS control, the combination of the three provided significantly more protection than rPep1, rPlb, or rAmn1 alone (fig. 11). This data is important in the search for the most immunogenic antigen to be used in a fungal vaccine because it combines antigens expressed at different stages of the parasitic cycle of *C. posadasii* (Tarcha *et al.*, 2006). This is an important aspect of a potential vaccine because a multicomponent vaccine would be able to provide protection at different stages of the pathogen's life cycle which in the host. Therefore, in creating a successful fungal vaccine, it may not be enough to include only one antigen. The research done by Tarcha *et al*. (2006) shows that it may be necessary to include multiple antigens which protect against all of the phases that a fungus may develop into to parasitize the host.

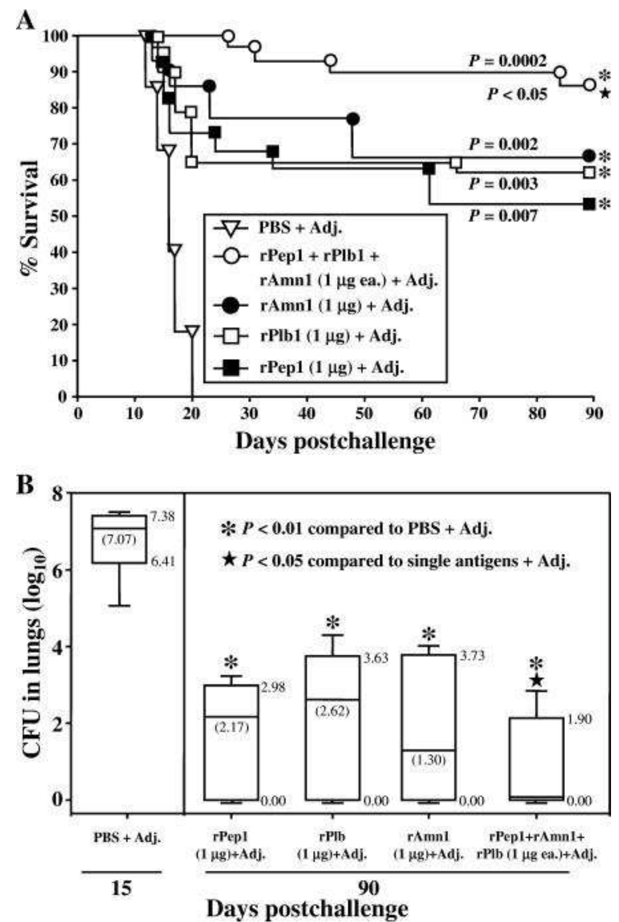

Figure 11: The three different antigens rPep1, rPlb, and rAmn1 were used to immunize mice separately and combined. **A.** Significantly more mice survived an infection with *C. posadasii* when protected by all 4 vaccines (rPep1, rPlb, rAmn1, and all 3 combined) compared to the control PBS, shown by the astericks. Also, significantly more mice survived infection when protected by all three antigens, as opposed to each antigen alone, shown by the star. **B.** CFU in the lungs is significantly less in mice immunized with rPep1, rPlb, rAmn1, and all 3 combined, shown by the astericks. Similar to A, all three antigens combined provide significantly more protection because there is significantly less CFU in mice immunized by all three antigens compared to each antigen alone, shown by the star.

Summary

Recent research on possible fungal vaccines has shown very important and helpful developments in the mechanism of vaccine protection. Preliminary research to isolate a soluble spherule wall fraction, particularly 27K vaccine, was crucial in revealing the protective ability of subunit vaccines, especially combined with the best adjuvant for vaccine release into the body (Zimmermann *et al.*, 1998). The identification of new immunogenic fungal antigens is one important development because, as the failure of the FKS vaccine revealed, it is most probable that isolation of known antigens from the nonantigenic spherule material can increase the immune response (Kirkland *et al.*, 1998). Kirkland *et al.* (1998), Dionne *et al.* (2006), and Tarcha *et al.* (2006) each found different fungal antigens, PRA, mannan, Pep1, Plb, and Amn1, isolated from *Coccidioides* spp. which could be used to create a fungal vaccine. Both Kirkland *et al.* (1998) and Tarcha *et al.* (2006) used murine models to test the efficacy of their proposed immunogens, and all antigens show protection for mice infected with *Coccidioides* spp. Identification of the fungal antigen is a critical step in the making of a fungal vaccine.

In addition to finding the best fungal antigen, Tarcha *et al.* (2006) show the importance of finding an antigen from each phase of the *C. posadasii* parasitic cycle. A combination of multiple antigens into one fungal vaccine has significantly more protection than each antigen alone (Tarcha *et al.*, 2006). This knowledge can be applied to the creation of other fungal vaccines, especially if the pathogenic fungus contains multiple phases of pathogenicity. Different antigenic proteins are expressed during different phases of pathogenicity, therefore, it is better to have protection against all phases that a fungus may develop into in the host rather just one possible phase (Tarcha, *et al.*, 2006).

The mechanism of vaccine protection was studied by Dionne *et al*. (2006), and the importance of maturation of dendritic cells into functional, antigen presenting cells is an essential element in vaccine efficacy. The adaptive immune response, and specifically the cell-mediated immune response, is one of the most important branches of the immune response in fighting off fungal infections (Heitman *et al.,* 2006). The findings of Dionne *et al.* (2006), which reveal the importance of dendritic cell antigen presentation of *C. posadasii* spherules, also show that a successful vaccine should probably target the cell-mediated branch of the immune system. While the necessity of fungal vaccines is a controversy still evident in many research institutions, these different research experiments show support in the search for fungal vaccines. While the profit of a successful fungal vaccine may not be as beneficial as some of the more widespread diseases which infect people, current research for fungal vaccines is still present and making breakthroughs in the creation of a fungal vaccine. Of these many breakthroughs, the combination of multiple immunogenic antigens, along with the best adjuvant, is the most promising candidate in the creation of a fungal vaccine.

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Aggressive Behavior of Male California Sea Lions during Breeding Season and the Effect of Interrelatedness on the Behavior of California Sea Lions at the St. Louis Zoo

Julia Goss and Professor Sussman
Behavioral Research at the Zoo

California sea lions' habitat ranges from the Pacific coast from Vancouver south to Baja California and the Gulf of California, but they breed all the way south to the Tres Marias Islands. Seven sea lions were observed in this study at the St. Louis Zoo, two males and five females. The group was observed for fifty five hours and their main activity cycle and social behavior patterns were recorded. The main results are as follows: The California sea lions spent the majority of their time resting with others (37%). RJ, the fertile male vocalized and played more aggressively than Woody the castrated male. Hide and RJ, mother and son, (on average) spent more time resting and swimming together than they did with any other sea lion. Hide and Seek, twin sisters, (on average) spent more resting and swimming together than they did with any other sea lion.

Introduction

The genus *Zalophus* is divided into three subspecies: *Zalophus californianus*, *Z. c. wollebaeki*, and *Z.c. japonicus* (Nowak 2004). The Japanese sea lion (*Z.c. japonicus*) was originally found in the Sea of Japan, but now is listed as extinct by the World Conservation Union (IUCN). The Galapagos sea lion (*Z.c. wollebaeki*) is found on the Galapagos Islands and off the coast of Ecuador and Colombia and is listed as vulnerable by the IUCN (Nowak 2004). The California sea lion (*Z.c. californianus*) is the only subspecies protected under law, the Marine Mammal Act of 1972, and remains off the endangered species list despite high mortality rates reported because of reduced food supplies due to El Niño (Nowak 2004). These sea lions' habitat ranges along the Pacific coast from Vancouver down through the Gulf of California and the coast of Baja California, and they will breed as far south as the Tres Marías Islands. Currently 110,000 sea lions are estimated to be located in California and 92,000 inhabit Mexico. In the spring, the males make annual migratory movements to breeding rookeries, often located in the Channel Islands and Mexico, while females remain closer to the breeding rookeries on the coast nursing their young for four to eight months (Riedman 1990).

California sea lions have brown coats, with the males usually being darker brown but having a wider range of pelage color, and the females being lighter, or "blonder" (Whitaker 1996). However, in the water their coats appear black. Their fusiform shaped bodies and foreflippers, which are modified forelimbs of land mammals used for swimming, allow them to dive up to 450 ft. They are capable of reaching speeds of up to 25 mph, the fastest of any aquatic carnivore (Whitaker 1996). Adult males have a raised forehead, or sagittal crest, that helps distinguish them from the females with lower, smoother foreheads. Adult males weigh between 440 and 860 pounds, while the females are much smaller, weighing 100 to 220 pounds (Whitaker 1996). These physical differences are examples of sea lion sexual dimorphism. Not only are the males twice as large, but their sagittal crest and wider range of pelage colors are secondary sexual characteristics (Scheffer 1969).

Male sea lions establish breeding territories from May to August. Males do not establish harems, a social unit maintained by a male fending off other males and preventing them from gaining access to other members of the harem (Bradbury 1981). Instead, sea lions exhibit resource defense polygyny or territorial defense. Because the females are not forced back into the territory, often called female defense polygyny, and there is more focus on the defense of the territory itself, it is correct to say California sea lions establish breeding territories, not harems. The term harem implies "exclusivity" (Grimzek 2004) between the males and females and, as Reidman states, the females can "exercise some choice" (1990) with whom to mate when in the breeding territories.

The gestation period of female sea lions is eleven months. The sea lions arrive at the breeding territory and give birth four to five days later. Three to four weeks after giving birth, the female will mate with one of the territorial males (Nowak 1999). The males will usually mate with around sixteen females in one breeding season. However, they do not remain in the rookeries, but migrate north to the coasts of California, Oregon, Washington and British Columbia. (Nowak 1999). The breeding territories also include bachelor groups of non-breeding males, juvenile males, and non-breeding juvenile females (Bradbury 1981). The composition of breeding territories is similar to the group of California sea lions living at the St. Louis Zoo.

The members of the order Pinnipedia, pinnipeds, or aquatic carnivores are considered the most vocal mammalian taxa. For that reason communication between the taxa, including the California sea lions, is often a subject of study

and experimentation (Schusterman et al, 2001). Both Schusterman et al (2001) and Peterson and Bartholomew (1967) agree that California sea lions vocalize more out of the water. However their vocalizations, similar to a “honking bark” (Whitaker 1996) have a wide variety of meanings. For instance, females use their signature vocalization to locate their pups in the rookeries. Males vocalize to show aggression, threaten other individuals, or attract a mate (Riedman 1990). These vocalizations, when the animals are congregated in a large group, can alert other sea lions of any type of danger (Bartholomew and Peterson 1967). The meanings behind these vocalizations can change, or vary in intensity depending on the season.

During breeding season, May to August, aggressive behavior is heightened in territorial males. This aggression is focused toward other males as a display of dominance over their breeding territories (Peterson and Bartholomew 1967). These behaviors can range from vocalized threats, a thrusting of the sea lion's head vocalizing with its mouth wide open, to fights that can inflict serious wounds because of the sea lion's sharp canines (Renouf 1991). During the breeding season the social structure is much more defined because the males must protect their rookeries. The level of dominance increases with the size of the male sea lion (Nowak 853). However, outside of breeding season the sea lions' social hierarchy is more lax because the males are not defending their breeding territories. The males also migrate northward after the breeding season, and their absence contributes to the lax social hierarchy.

The current study was conducted during the breeding season, at the sea lion tank at the St. Louis Zoo which has one juvenile male capable of breeding and one fully adult castrated male. It can be hypothesized that the juvenile male would vocalize more and show increased aggressive behavior as opposed to the castrated male.

Another topic that has been extensively studied is the imprinting propensity of the sea lion pup, especially to humans. During various experiments, if the sea lion was reared by a surrogate human and then allowed to return to its biological mother or its zookeeper, it usually preferred its surrogate mother (Reidman 1990) and could even recognize him/her up to five years later. This behavior also was found to be true of captive California sea lion pups with their mothers. In captivity, the sea lion pups not only interacted more with their mothers after a long period of separation but also interacted more with sea lion kin than with unrelated sea lions. While it would seem that a sea lion pup in the wild would recognize its mother after long periods of separation as it does in captivity, this ability has yet to be observed (Riedman 1990). Based on the family relationship of the sea lions at the St. Louis Zoo and the previous experiments concerning kin relations, one would expect the two female twins and one of their sons to interact more frequently than the remaining unrelated sea lions in the tank.

For this study, I observed seven California sea lions, two males and five females. I monitored their general social behaviors and to what extent their interactions were friendly or aggressive. I also recorded the location of their habitat in which these behaviors occured, with whom they occured, and when they occured. Specifically, I studied the differences in vocalization and aggressive behavior in the juvenile male versus the castrated male. Additionally, because of the interrelatedness of three of the sea lions I determined whether or not there are a greater number of interactions between related individuals or between these individuals and unrelated sea lions.

Methods

The Saint Louis Zoo has seven California sea lions that are housed separately from the sea lions that perform in the shows. The tank group consists of two males; RJ, the juvenile male, and Woody, the castrated fully adult male. There are five females including two fertile twins, Hide and Seek. All of the females are capable of reproduction (Figure 1).

Name of Animal	Sex	Date of Birth	Other
Woody	Male	6/12/86	Castrated Male
RJ	Male	6/27/03	Juvenile Male - fertile
Hide	Female	6/21/90	Dominant female
Seek	Female	6/21/90	Dominant female
Ethel	Female	6/10/87	Third highest female
Patches	Female	6/02/87	Fourth highest female
Julie	Female	6/23/92	Fifth highest female

Figure 1: Names, Sexes, Date of Birth, and other information concerning the California sea lions

The social hierarchy, although not completely rigid, has seventeen year old Hide and Seek as the dominant females, twenty year old Ethel as the third highest, twenty year old Patches as the next lowest, and fifteen year old Julie as the lowest ranking female. Julie is the smallest and youngest. The twenty one year old male, Woody, is dominant over the juvenile four year old RJ, although he has a more relaxed demeanor. Each sea lion with the exception of Patches, had either one or both parents born in the wild (Figure 2).

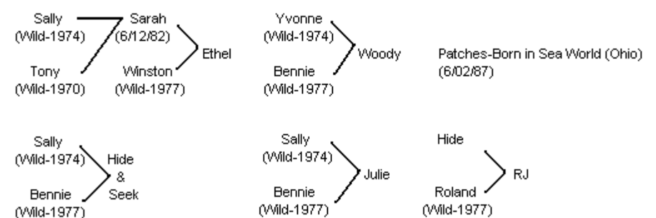

Figure 2: Genealogy trees of the seven sea lions

Caging

The sea lions are housed in a tank on the south side of the zoo. The tank is nine feet deep and holds 200,000 gallons of fresh water to which salt is added. The tank forms a

figure eight, with smooth floors and walls, except for the two rock formations on both sides of the tank. These rock formations are constructed with various ledges and crannies that offer places to rest and receive food during feeding times. The formations were made to mimic the rocky gravel beaches that the females use as rookeries. The tank is cleaned every Thursday. The cleaning consists of draining the tank, removing the algae on the walls and floor of the tank with a power hose, and refilling it.

There are few objects for enrichment in the tank, as wild sea lions do not play with objects; however, a blue ball is often floating around in the water for the sea lions to play with, if they so desire.

As far as recording where the specific animals were located while collecting the data, the tank was divided into seven sections, L (Land) 1-3 and W (Water) 4-7 (Figure 3). The land levels represented the sections of rock in the tank. Land 1 was the uppermost level located on the east side of the tank, and Land 2 was the one underneath it. Land 3 was on the west side of the tank. Land 2 and 3 were then divided into 2A and 2B and 3A and 3B, with the A sections being on the north side of the tank, and the B sections being on the south side of the tank. The water sections of the tank were then divided into quadrants, 4 being the Northwest quadrant, 5 the Northeast quadrant, 6 the Southwest quadrant, and 7 the Southeast quadrant. The sea lions would very rarely exit the water on the edge of their tank so these sections were labeled with the direction they were facing when exiting, N north, S south, E east, W west (Figure 3). The sea lions were usually all visible at once unless resting on opposite sides of the rocks, in which case moving around the exhibit allowed one to locate all of them.

Materials

Two data sheets were used to collect the maximum amount of information concerning social behaviors. The first was a scanning data sheet which was used to record the sea lions' behavior, location, and nearest neighbor every ten minutes (Figure 4). The behaviors were recorded using the legend derived from the ethogram of mutually exclusive behaviors (Figure 5). The area in which each individual was located was recorded with a tally mark under the appropriate section. The first letter of the sea lions' name was used to represent whichever sea lion was the nearest neighbor. The second data sheet was used to monitor a focal animal (Figure 6). For one hour increments a sea lion was chosen at random and its social behaviors, duration of behavior, and location were recorded. The focus here was strictly on the social behaviors of an individual sea lion. The same ethogram used in the scanning sample was used in the focal sample, however, only the social interactions were recorded. These interactions included vocalizing, playing, swimming, resting, or jugging with other sea lions. Each vocalizing or playing interaction between the focal individual and another sea lion was recorded as either friendly or aggressive. This data sheet helped to provide more detailed information on the sea lions' social behavior (Figure 6).

A stopwatch, pen, clipboard, and a spiral notebook for ad lib notes were used during the study.

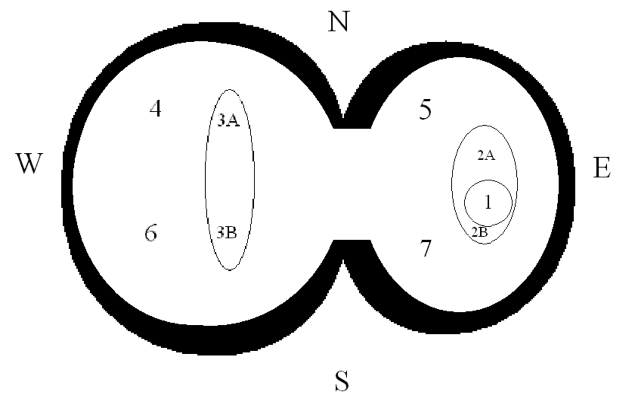

Figure 3: 1-3B represent the five sections of rock in the tank, 4-7 represent the sections of water, and N,S,E,W are the directions the various sections are facing.

Date: 6/7/07	Woody	Ethel	Hide	Seek	Patches	Julie	RJ
Time: 12:00							
Behavior:	SO	SO	SA	RO	P	RO	P
Nearest Neighbor	E	W	-	J	RJ	S	P
Location:	7	7	4	2A	5	2A	5

Figure 4: Sample data sheet for focal sampling. Data was recorded every 10 minutes.

Behavior	Description	Behavior	Description
Swimming with Others (SO)	Two or more individuals moving forward in the water using their flippers to propel themselves without porpoising	Jugging (J)	Both hind flippers and one foreflipper extend above the surface (Bearzi 2006)
Swimming Alone (SA)	One individual moving forward in the water using his/her flippers to propel itself without porpoising	Porpoising (PO)	Leaping from the water and reentering headfirst
Resting Alone (RA)	One individual that remains stationary and is located more than one meter away from another individual	Vocalizing (V)	Making any sort of vocal noise whether it is a grunt, bark, or growl
Playing (P)	Two or more individuals in the water interacting closely in a friendly or aggressive manner	Resting with Others (RO)	Two or more individuals that remain stationary and are located within one meter of each other or are touching another individual
Feeding (F)	All individuals are stationed in their feeding zones and are receiving hand tossed fish	Swimming Congregated (C)	All sea lions congregated in one swimming area either moving in a circuit around the tank or remaining in that area

Figure 5: Ethogram of mutually exclusive behaviors used in the scan and focal sampling.

Time	Actor	Action	NN	L1	L2	L3	W4	W5	W6	W7	F	A	Date:
11:50-12:30	RJ	SO	H				I						6/7/07
12:30	RJ	V	H					I			I		
12:30-12:33	RJ	P	W				I				I		

Figure 6: Sample of focal data sheet, illustrating the behaviors swimming with others, vocalizing, and playing determined by Figure 4.

Results

The California sea lions' activity cycle at the St. Louis Zoo consisted mainly of Resting with Others, which occurred 37% of the time, Swimming Congregated (17%), Swimming Alone (14%), Swimming with Others (13%), and Resting Alone (11%). Less frequent activities included Jugging (4%), Playing (2%), and Vocalizing and Feeding (both 1%) (Figure 7).

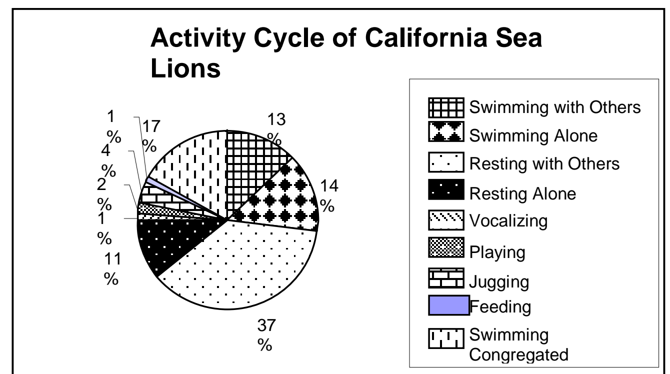

Figure 7: Activity cycle of the California Sea Lions at the St. Louis Zoo

The sea lions spent nearly equal time in the water (52%) and out of the water (48%). However, they did spend noticeably more time in certain water and land sections (Figure 8).

In the water sections, the sea lions swam in Section 4 the most at 23% and Section 6 at 16%. These frequencies of tank use were most likely related to the fact that the zookeeper entered this section of the tank for each feeding. The sea lions would congregate up to an hour before feedings in those two sections anticipating the zookeeper's arrival. Sections 5 and 7 were not near the zookeeper's entrance and therefore less swimming occurred in these sections. As far as land sections, resting in Sections 1 was the most frequent (18%), then 3B (16%). However, the frequency of resting on land reflected each sea lion's resting spot preference. Most had a certain spot on which they would rest when they were out of the water for a long period of time. Section 1 was possibly the most frequently visited because between Woody and Ethel the average number of times each rested on Section 1 was 148 times during the observation period, while for the remaining five sea lions the average number of times each rested on Section 1 was 113 times.

While resting and swimming with others occurred more frequently as social behaviors within the group, playing bouts and vocalizations were categorized as either aggressive or friendly. There were more friendly playing bouts (35) than aggressive playing bouts (12). However, there were more aggressive vocalizations (72) than friendly vocalizations (50) (Figure 9).

One reason for the predominance of aggressive vocalizing was in defense of RJ's (the 4 year old fertile male) aggressive behavior. From the total of 56 aggressive vocalizations recorded (excluding RJ's), 27 or 48% were directed towards RJ (Figure 10). Patches was the source of nearly all these aggressive vocalizations toward RJ.

RJ's social behavior while playing and vocalizing was significantly more aggressive than Woody's (the 21 year old castrated male). While Woody emitted only 2 aggressive vocalizations, RJ emitted 18. Woody never played aggressively, while RJ did so 7 times (Figure 11).

Interactions between RJ and Hide (son and mother) and Hide and Seek (twin sisters) indicated that their familial relationship corresponded positively with how much time they rested and swam together, but had a negative relationship with the amount of time they spent in playing and vocalizing with one another.

RJ and Hide played slightly less than RJ and the others. RJ played 7 times with Hide, while playing an average of 7.8 times with the other sea lions (Figure 12). An even greater difference was observed between the number of RJ's vocalizations toward his mother and those toward other sea lions. RJ and Hide vocalized 4 times with each other, while RJ vocalized an average of 17.8 times with the other sea lions. However, RJ and Hide spent much more time swimming and resting together than RJ did with the other sea lions.
RJ and Hide swam together for 181 minutes, while RJ swam with others for an average of only 19 minutes. RJ and Hide rested together for 107 minutes, and RJ rested with others for an average of 23 minutes (Figure 13).

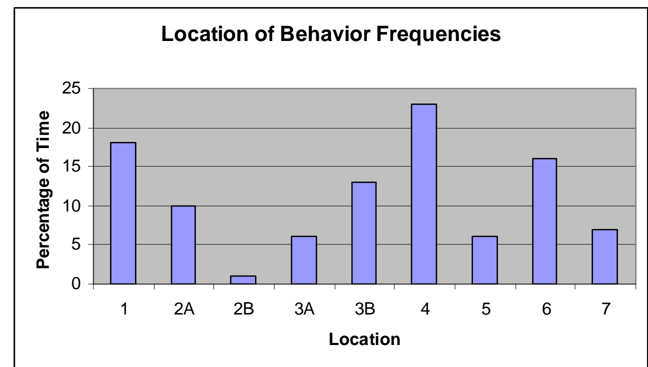

Figure 8: Time spent in each section of the tank

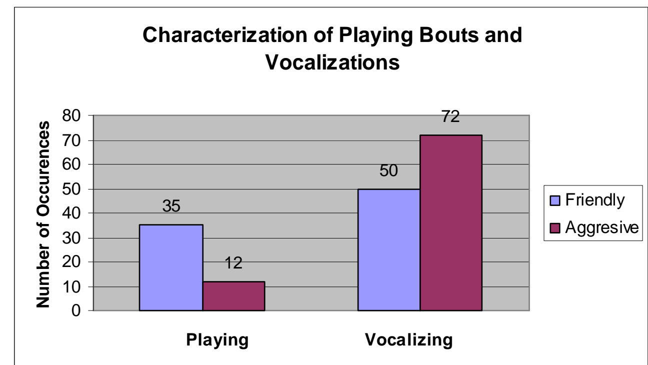

Figure 9: Number of friendly and aggressive playful bouts and vocalizations

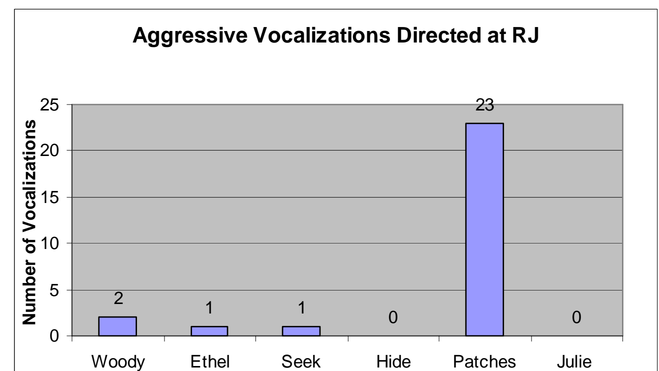

Figure 10: Aggressive vocalizations directed at RJ

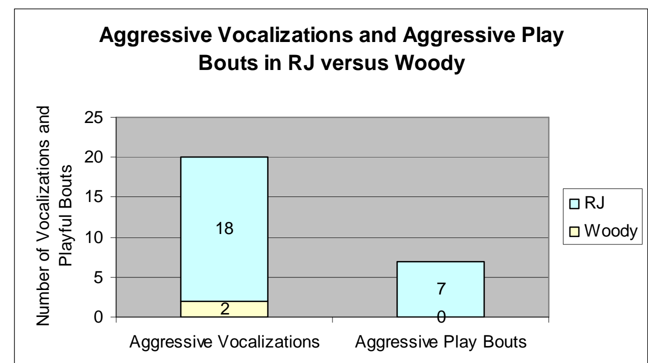

Figure 11: Number of aggressive vocalizations and play bouts in RJ versus Woody

Interactions between Hide and Seek mirrored those of RJ and Hide. Hide and Seek never played together, while Hide played with others an average of 1.4 times. Hide and Seek also never vocalized with each other, while Hide vocalized with each of the others an average of only one time (Figure 14). However, Hide and Seek swam together for 77 minutes, and Hide swam with others (excluding RJ her offspring) only an average of 20.6 minutes. Hide and Seek rested together for 120 minutes, while Hide rested with others (excluding RJ) an average of 51 minutes (Figure 15).

Discussion

During this study I investigated the St. Louis Zoo sea lions' general behavior patterns, as well as the difference in behavior between a fertile male and a castrated male, and the differences in behavior among those with familial relations. The sea lions spent the majority of their time interacting socially, whether resting, swimming, or jugging together, or playing or vocalizing. The predominance of social behavior supports Miller's (1991) claim that pinnipeds make ideal subjects because of the researcher's ability to study them at close range and the sea lions' high frequency of communication and social interaction. However, as Riedman (1990) points out, sea lions' social organization in the wild changes seasonally. These changes involve a more structured social hierarchy during breeding season while the male protects his rookery, and a less strict hierarchy during non-breeding season. Because the sea lions at the St. Louis Zoo are contained within a tank and I did not observe them out of breeding season, watching their social organization change was impossible. Also, due to RJ being the only male capable of breeding made determining the social structure during the breeding season difficult because he had no other fertile males with whom to interact.

Schusterman (2001) states that pinnipeds are considered to be the most vocal mammalian taxa. The sea lions at the St. Louis Zoo are no exception and their high frequency of vocalization helped me discern behavioral differences between a male sea lion in breeding season and one who is not breeding. By categorizing their vocalizations as either friendly or aggressive I was able to discern the differences between Woody and RJ's behavior. RJ acted as a fertile male would in breeding season; however, because Woody is castrated he acted as a sea lion would out of breeding season. RJ's behavior involving playing and vocalizing was much more aggressive than Woody's behavior in these activities. RJ's aggressive behavior also affected the behavior of the five other females in the tank. The majority of the females' vocalizations were directed at RJ in response to his aggressive behavior. Patches, in particular, was a target of RJ's aggressiveness, which is reflected in her high frequency of aggressive vocalizations toward him. The high level of aggressive behavior in RJ as opposed to Woody supports Peterson's and Bartholomew's (1967) findings that aggressive behavior is more developed during the breeding season. There exists, however, a great discrepancy in age between Woody (21) and RJ (4). Further research between breeding and non-breeding males of similar ages would clarify whether this type

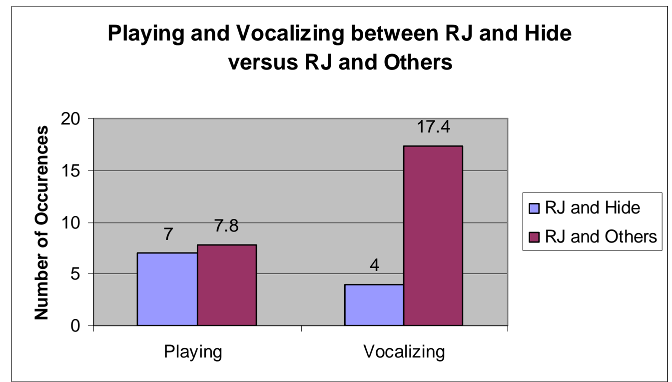

Figure 12: Playing and vocalizing occurrences of RJ and Hide

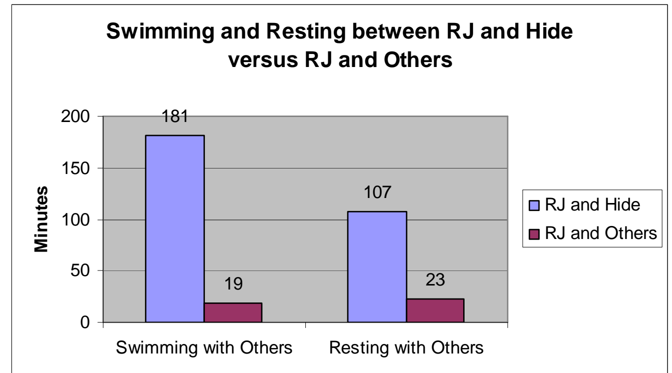

Figure 13: Swimming and resting time of RJ and Hide

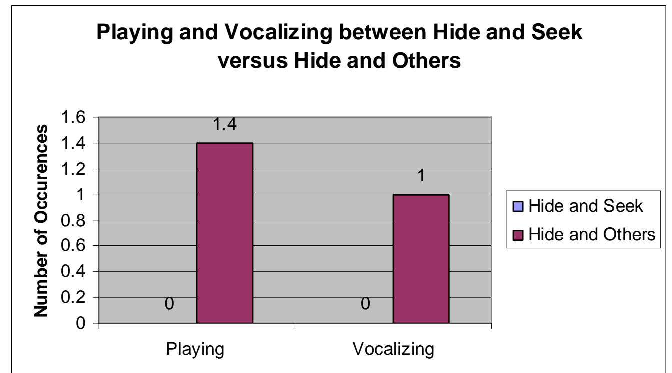

Figure 14: Playing and vocalizing occurrences of Hide and Seek

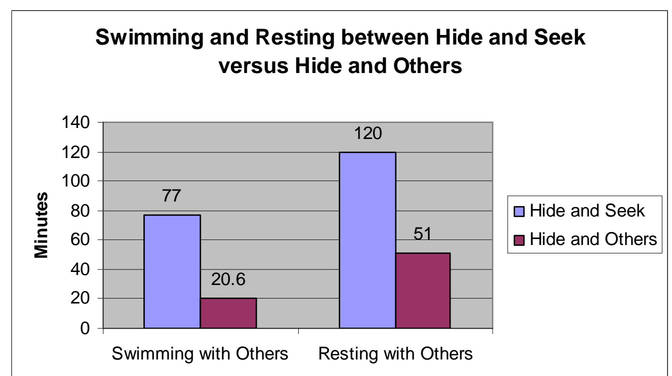

Figure 15: Swimming and resting time of Hide and Seek

of behavior is due primarily to breeding status or if age is a factor in this difference in aggressiveness.

Observations of the St. Louis Zoo sea lions support Riedman's (1990) conclusions regarding interactions between sea lion kin. Riedman's finding that mothers and their sea lion pups born in captivity interacted more with each other than with unrelated sea lions was supported, to some extent, by Hide and RJ's relationship. Although the two vocalized and played with each other less than they did with the other sea lions, they both spent greater time on average swimming and resting together than with any of the other sea lions. Given that the majority of vocalizations recorded in this study were aggressive in nature, it is not surprising that few vocalizations occurred between RJ and Hide, whose relationship was observed to be friendly in nature. Riedman's observation that sea lion kin spend more time interacting was also supported by the data regarding Hide and Seek's interactions. Hide and Seek, like Hide and RJ, vocalized and played less together than with the other sea lions, but they did spend more time on average swimming and resting together than Hide did with the other sea lions. While Riedman points out these strong kin relations have not been observed in the wild, they are apparent in captivity among the sea lions at the St. Louis Zoo.

As previously mentioned more research is needed to determine the relationship between aggressive behavior and age versus breeding status among male sea lions in captivity. In the future, I would study males of similar age both in and out of breeding season. In addition, during this study two sisters were observed and conclusions were drawn regarding social behaviors among related sea lions. However, relations between two male siblings or a male and female sibling could be studied to determine if these relationships were similar to those found between the two sisters. During this study a mother-son combination was observed. Future studies should include observing the quality of mother-daughter relationships to see if they are similar to the mother and son relationship.

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A novel connection between the Nuclear Pore Complex and the Cell Division Cycle in *Saccharomyces cerevisiae*: The Nucleoporins Nup84 and Nup188 are significant to the Activity of the G1 Cyclin Cln3, while Nup2 is not

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This investigation offers the first documentation of a physiological link between the function of the G1 cyclin Cln3 and the nuclear pore complex (NPC). In Saccharomyces cerevisiae, commitment to cell division is largely regulated by a complex of two proteins, Cdc28 and Cln3. This complex must be relocated from the cytoplasm to nucleus, where the genetic material is housed. Though a number of additional proteins are thought to support this movement, none have previously been identified. Three proteins of the NPC, the channel through which the complex is thought to pass to enter the nucleus, were previously defined as important in the localization of an artificial construct consisting of a portion of the Cln3 protein. This research identifies genetic interactions between full-length Cln3 and NUP84, NUP188, and NUP2 to ascertain the relevance of these nucleoporin-encoding genes in regulated cell division. Cln3 activity is reduced in the absence of NUP84 and NUP188, but not in the absence of NUP2. The absence of NUP188 increases the relative frequency of unbudded cells, which suggests that the lack of this NPC component delays passage through the G1 phase of the division cycle. The absence of NUP84 does not increase the frequency of unbudded cells, but rather increases the frequency of cells with aberrant morphologies. The investigation concludes that NUP188 is significant to the functionality of Cln3, while NUP2 is not. Data suggests that NUP84 assists in Cln3 function, but further investigation is required to establish the effect this has on cell cycle progression.

Introduction

The division cycle of eukaryotic cells is a highly regulated process that involves the duplication of a cell's components and its subsequent separation into two daughter cells. In order for the daughter cells to be both functional and genetically identical to their predecessor, the mother cell must utilize methods that insure the accurate duplication and/or segregation of its genetic material (chromosomes), organelles, and other cellular components. These methods are collectively called the cell-cycle control system. This system employs a reliable series of molecular activities at the beginning of each cell cycle to ensure the proper coordination and chronology of events (Morgan 2007).

The cell-cycle control system is primarily dependent on a group of proteins known as cyclin-dependent kinases (Cdks). In order for Cdks to become active, they must be bound by a member of a family of regulatory proteins known as cyclins. Cyclins activate the Cdks by causing a conformational change in the Cdk protein structure and targeting the kinase to appropriate substrates (Morgan 2007). The cellular concentration of Cdks remains fairly constant throughout the cell cycle, so the cell-cycle control system relies on the oscillation of different cyclin proteins to initiate particular events. Thus, cyclin-Cdk interactions provide the bulk of the regulatory mechanisms as the cell progresses through its cycle (Morgan 2007).

To maintain the proper functioning of cyclin-Cdk units, cells implement a series of checkpoints at certain transitory periods in the cell cycle. One major checkpoint in the cell cycle, called "Start", occurs as cells pass out of G1 and move into S phase. This regulation is crucial because passage through Start irreversibly commits a cell to the division process. If conditions (both intracellularly and environmentally) are not favorable for division, certain signals may act to inhibit Cdk activity, thus preventing the cell to progress through the Start. When defective, this checkpoint plays an important role in the development of oncogenic properties in mammalian cells (Biggs & Kraft 1995).

The budding yeast *Saccharomyces cerevisiae* utilizes a single Cdk, called Cdc28, throughout the cell cycle. The precise function of the Cdk is dictated by the particular cyclin which is present in the complex. Though transition through the Start checkpoint is dependent on both G1 and G1/S cyclins, it is believed that the G1 cyclin Cln3 normally initiates the transcription events necessary for this progression. Progression through Start requires that the Cln3-Cdc28 complex is able to initiate transcription during G1 by acting on a transcriptional regulatory network. Since metabolism and nutrient availability are directly indicated in the growth rate of the cell (and thus its potential to initiate division), Cln3 concentrations may act as an indicator of a cell's readiness to enter the division cycle (Miller & Cross 2000, Morgan 2007).

Cln3-dependent progression through the Start checkpoint requires that the Cln3-Cdc28 complex first pass from the cytoplasm (where it is made) into the nucleus (where it functions) (Futcher & Edgington 2001, Miller & Cross 2000, Miller & Cross 2001). Passage into the nucleus is energy dependent and requires Ran GTPase, consistent with classic import via the nuclear pore complex (NPC) (Miller & Cross 2001). Once the Cln3-Cdc28 complex is within the nucleus, it is able to indirectly initiate the transcription of G1/S transition genes by acting on a regulatory network (Costanzo et al. 2004, Levine et al. 1996, Miller & Cross 2001).

The nuclear pore complex serves as the primary transport channel for macromolecular traffic between the nucleus and cytoplasm of a cell. In *S. cerevisiae*, the NPC is thought to be comprised of approximately 30 distinct proteins, collectively named nucleoporins (Adam 2001). The NPC spans the width of the nuclear envelope and can be categorized into three distinct components: an octagonal nuclear core, a cytoplasmic ring equipped with filaments, and a nuclear basket (Suntharalingam & Wente 2003).

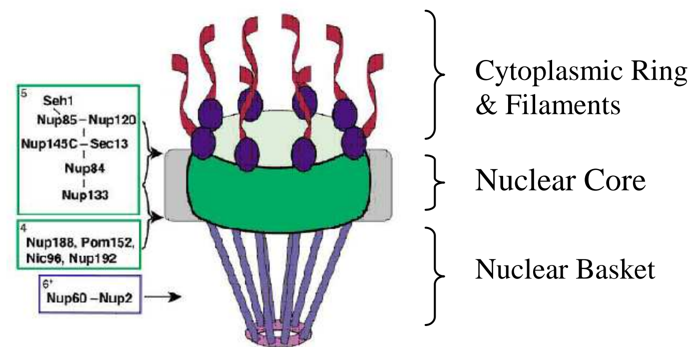

Figure 1: Nup2, Nup84, and Nup188 belong to separate subcomplexes within the nuclear pore complex. The center figure represents a simplified rendering of the nuclear pore complex in *S. cerevisiae*. The boxes on the left represent the three subcomplexes to which Nup84, Nup188, and Nup2 (top to bottom) belong. Dashes between protein names indicate that the proteins display defined interactions with one another. Commas between protein names indicate that the associations are not well understood. Complexes containing dynamic nucleoporins are indicated by an asterisk. General structure of the NPC structure is indicated on the right (Modified from 9).

Small molecules (<30-40 kiloDaltons) may diffuse freely through the NPC, but larger molecules must be actively transported through the channel (Adam 1999, Adam 2001, Fabre & Hurt 1997). Recent examination of the NPC suggests that it functions not only in molecular trafficking, but also in spindle and kinetochore assembly, chromatin organization, and transcriptional silencing (Siniossoglou et al. 1997, Suntheralingam & Wente 2003).

An additional protein family, individually labeled as either importins or exportins, mediates the active transport through the NPC. As their names imply, importins facilitate the nuclear localization of proteins and exportins shuttle proteins from the nucleus into the cytoplasm (Adam 1999). Proteins that require nucleocytoplasmic transport often contain specific amino acid sequences that allow importins and exportins to recognize the protein cargo. These sequences may be classified as either nuclear localization signals (NLS) or nuclear export signals (NES), depending on their function. Currently, the two categories of NLS that are best understood are the SV40 type and the bipartite type. The SV40 NLS type is characterized as a short cluster of positively-charged residues, while the bipartite type consists of two positively-charged basic clusters separated by a linker region. Cln3 contains a bipartite type NLS at the C-terminus, which has been identified as extremely important for the regulation and nuclear localization of Cln3 (Futcher & Edgington 2001, Miller & Cross 2001).

While the bipartite NLS of Cln3 has been clearly defined, the genes that support the passage of Cln3 into the nucleus have not been distinctly identified. A genetic screen was performed to determine those cellular genes that impact the movement of Cln3 into the nucleus. To facilitate the analysis of Cln3 nuclear localization, the screen utilized Green Fluorescent Protein (GFP)-based live cell imaging. Unfortunately, complications arise in attempting to determine the cellular location of full-length Cln3 protein that has been tagged with a GFP marker. The high propensity for Cln3 to degrade, coupled with its low abundance in wildtype strains, inhibits the detection of GFP-tagged Cln3 using current methods. Therefore, a GFP fusion that utilizes only the Cln3 NLS sequence was used in the screen. The Cln3 NLS-GFP fusion was expressed from a high copy plasmid in the cell to allow efficient detection of the cellular localization of the Cln3 NLS (Figure 2A). This method provides an effective and straightforward assay for Cln3 NLS activity.

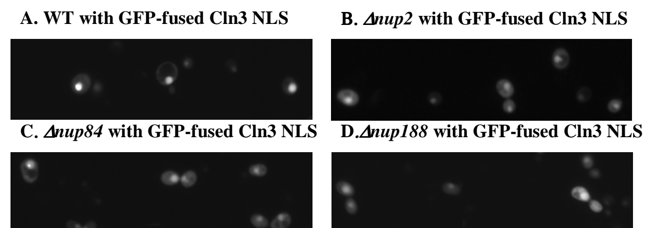

Figure 2: Cln3 NLS defects are observed to a degree in ***Δnup2, Δnup84,*** and ***Δnup188*** strains. *S. cerevisiae* cells expressing the GFP-tagged Cln3 NLS. A) 1254-14D (WT), B) MMY133-5A (*Δnup2*), C) MMY131-12C (*Δnup188*), and D) MMY135-8D (*Δnup84*) show varying levels of NLS activity.

Seventy nine genes in *S. cerevisiae* annotated as important for the nucleocytoplasmic transport of macromolecules by the *Saccharomyces* Genome Database (accessed 2005) were screened for their relevancy in the nuclear localization of the Cln3 bipartite NLS, using the GFP-tagged Cln3 NLS localization screen. Deletion strains were recorded based on their GFP localization defects and eleven of the assayed genes (*KAP114, MLP2, MFT1, NPL3, NUP2, NUP84, NUP188, TEX1, THP2, UBS1*, and *THI73*), were identified as important for Cln3 NLS activity (Miller, raw

data). Of these eleven genes, *NUP84, NUP188,* and *NUP2* encode components of the nuclear pore complex.

The Nup84 protein is a nucleoporin that is located on both the cytoplasmic and nuclear peripheries of the NPC as a component of the Nup84 subcomplex (Figure 1) (Suntharalingam & Wente 2003). The Nup84 subcomplex on the nuclear face of the NPC has been suggested to function in the reverse recruitment model for gene activation (Menon et al. 2006). In this model, the Nup84 subcomplex serves as a protein platform to which transcriptional coactivators (Rap1/Gcr1/Gcr2) are anchored (Menon et al. 2006). Activators may then shuttle the DNA to this anchored structure for transcription (Menon et al. 2006). Nup84 interacts with two proteins, Spo7 and Nem1, to aid in the proper construction of the nucleus (Siniossoglou et al. 2000). Consequently, aberrant morphology of the nuclear envelope is often displayed in Nup84-null cells, which may impact the passage of proteins through the NPC (Adam 2001, Sinniossoglou et al. 2000).

The gene *NUP188* was also identified as important for Cln3 NLS activity by the GFP-tagged Cln3 NLS screen. Nup188 is thought to cooperate with the nucleoporins Nic96, Pom152, Nup157, and Nup170 in forming the structural core of the NPC (Figure 1) (Adam 2001). *NUP188* deletion strains display aberrant nuclear envelope morphology and defects in the nuclear localization of proteins (Adam 2001). The importance of Nup188 to the proper localization of the Cln3 NLS may imply that the aberrant morphology of the nuclear envelope in the deletion strain only impacts the nuclear import of a select set of proteins.

The third nucleoporin that the genetic screen identified as important for Cln3 NLS activity is Nup2. The Nup2 protein has been characterized as a component of the nuclear basket of the NPC (Figure 1) (Adam 2001, Suntharalingam & Wente 2003), but its recent implications as a dynamic protein suggests that it also functions on the NPC's cytoplasmic face (Dilworth et al. 2001). When Nup2 is localized to the nuclear basket, it is observed to aid in the regulation of gene expression through the generation of chromatin boundaries (Brown & Silver 2007, Dilworth et al. 2001).

The goal of this research is to determine the physiological relevance of the Nup84, Nup188, and Nup2 genes in relation to full-length Cln3 function. Though these proteins are clearly identified as important for Cln3 NLS activity as determined by live cell imaging of the GFP fusion, it is not yet established that they affect the ability of full-length Cln3 to properly support progression through the Start checkpoint. Currently, no published literature has established a link between structural components of the nuclear pore and regulation of the cell division cycle in *S. cerevisiae*. If the proposed research is successful, it may provide a novel connection between two subdisciplines of cell biology.

Methods

The strain 1254-14D is congenic with BF264-15D: *MAT***a** *trp1 leus ura3 ade1 his2* and was constructed as described previously (Miller & Cross 2001). The experimental strains MMY135-8D (Δ*nup84*), MMY133-5A (Δ*nup2*), and MMY131-12C (Δ*nup188*) were constructed by Mary Miller via crossing strain 1254-15D (Table 1) and S288C strains containing specified gene deletions (Open Biosystems Inc.).

The plasmids pMM82 and pMM99 support expression of *CLN2* and *CLN3,* respectively. The plasmids each contain a 9X myc epitope-tagged *CLN* gene under the control of the *CLN3* promoter in a low-copy-number *TRP1* vector (Miller 2001). Isolation of the plasmids was carried out with the QIAprep Spin Miniprep Kit as directed (Qiagen Inc.). Preparation of bacteria and yeast media and construction/ maintenance of plasmids were carried out as described previously (Miller & Cross 2000). Plasmid based yeast transformations were carried out using the Frozen Yeast Transformation II kit (Zymo Research Inc.).

Ten-fold serial dilutions of yeast cells for the viability assay were performed as described previously (Miller & Cross 2000). Three µl of each dilution was transferred in duplicate to a complete agar media containing either galactose (YPG) or dextrose (YPD) as the sole carbon source. A single plate from each duplicate was then incubated at either 30° or 38° C until colonies were readily visible (36 to 48 hours) (Miller & Cross 2000). Viability assays were repeated a minimum of three times on independent transformants for each of the yeast strains employed in this research.

Table 1. Strains used in this study

Strain	Relevant Genotype
1254-14D	*MAT***a** *cln1*Δ *cln2*Δ *cln3*Δ *leu2::LEU2::GAL1::CLN1*
MMY135-8D	*MAT***a** *cln1*Δ *cln2*Δ *cln3* Δ *leu2::LEU2::GAL1::CLN1 nup84*Δ
MMY131-12C	*MAT***a** *cln1*Δ *cln2*Δ *cln3* Δ *leu2::LEU2::GAL1::CLN1 nup188*Δ
MMY133-5A	*MAT***a** *cln1*Δ *cln2*Δ *cln3* Δ *leu2::LEU2::GAL1::CLN1 nup2*Δ
S288C	*MAT*α *CLN1 cln2*Δ *CLN3* trp1 ura2 his2 leu2

Table 1: The 1254-14D strain is congenic with BF264-15D: *MAT***a** *trp1 leus ura3 ade1 his2.* All other strains were constructed by Mary Miller via crossing strain 1254-15D and S288C strains containing specified gene deletions (Open Biosystems Inc.).

To establish the budding index, pMM82 (Cln2-dependent) or pMM99 (Cln3-dependent) transformant cells were inoculated in triplicate into 5 ml of YPD liquid media. These cultures were incubated in a shaker at 38° C until they reached an optical density of 0.4 to 1.0, which indicates that the cell population is in a logarithmic growth state (12 to 18 hours). Upon reaching the correct optical density, cells were harvested by centrifugation and resuspended in 900 µl of phosphate buffer (13.6 g Anhydrous KH2PO4, 2.1 g KOH, 0.5 mM MgCl2, up to 1 liter with distilled water) and fixed with 100 µl of formaldehyde (Fisher Scientific) for a length of 5 minutes. Following, the samples were resuspended in 1 ml of phosphate-buffered saline (PBS, Hyclone) and sonicated for 10 seconds each. The samples were then resuspended in 100 µl of PBS and 3 µl of each sample was prepared on a slide for observation at 40x with an Olympus CX31 transmitted-light microscope.

The cells within each field of view were thoroughly counted and qualified as either budded, unbudded, or aberrant. Aberrant cells were defined as displaying morphologies uncharacteristic of wildtype yeast cells, such as irregular processes, chains of cells that did not complete cytokinesis, or irregularly-shaped cells (Figure 5). A minimum of 300 cells from each sample were characterized, resulting in a total of at least 900 cells for both Cln2-dependent and Cln3-dependent populations from each yeast strain. The data sets from each cell population were utilized in calculating the average frequency of unbudded cells and graphing these values with a 2x standard error bar for each set (calculated as [standard deviation x√3]x2). This index was reproduced twice more from two additional sets of transformants. Comparison between the frequencies of unbudded cells was then used to determine if the Cln3-dependent cells from any of the deletion strains displayed significant deviations from WT 1254-14D relative to the Cln2-dependent cells.

RESULTS & DISCUSSION

To address the importance of Nup2, Nup84, and Nup188 in the proper functioning of full-length Cln3, the research has taken a two-pronged approach. The first attempt to establish relevancy between Cln3 and the specified nucleoporins consisted of a viability assay (Figure 3), in which the functional ability of G1 cyclins is assayed in mutant cells (lacking either *NUP2, NUP84,* or *NUP188*). When the *CLN3* gene is deleted, Cln1 and Cln2 are capable of rescuing Cln3 function and allowing the cell to progress through the cell cycle (Futcher & Edgington 2001, Levine et al. 1996). Thus, the presence of Cln1 and Cln2 may mask defects in Cln3 activity. However, the genes encoding these cyclins are collectively essential and at least one of the cyclins must be present to ensure a cell's viability. To resolve this issue, *CLN1*, *CLN2* and *CLN3* are deleted from the yeast strains used for viability assays and *CLN1* is placed under an inducible *GAL1* promoter (Table 1).

Either *CLN2* or *CLN3* may then be introduced into the strain via a plasmid transformation and *CLN1* expression may be inhibited by withdrawing the galactose carbon source. Furthermore, expression of *CLN1* may be actively repressed by the presence of dextrose as the sole carbon source. In theory, the cells inoculated onto the complete galactose media should be equally viable, regardless of the plasmid with which they were initially transformed. Thus, the cells on the galactose media served as a control when compared to the cells inoculated onto dextrose media by insuring that the number of cells utilized from each tester strain was relatively uniform.

While the redundancy of Cln1 and Cln2 complicates the construction of experimental strains, their ability to rescue Cln3 function aids in determining the Cln3 specificity of functional defects that we observe. While Cln3 activity requires nuclear localization, Cln2 activity does not (Futcher & Edgington 2001, Miller & Cross 2001). Additionally, Cln2 does not contain any obvious NLS regions and should be able to function independently of any localization factors identified in our screen (Futcher & Edgington 2001, Miller & Cross 2001). By experimentally comparing the activity of a Cln3-dependent strain to a Cln2-dependent strain, it was possible to identify defects specific to Cln3. If the gene in question specifically influences the activity of full-length Cln3, then the Cln3 transformants should display a slower growth rate relative to the Cln2 transformants.

Cln2 and Cln3 were comparable in their capacity to rescue the viability of the wildtype *NUP* tester strain (1254-14D) when *CLN1* was no longer expressed. The deletion of *NUP84* produced an observable reduction in the growth of Cln3-dependent cells in relation to Cln2-dependent cells in the viability assay (Figure 3). The Cln3-dependent *nup84* cells display a slight but reproducible reduction in viability in comparison to the Cln2-dependent *nup84* cells. Since Cln3 must achieve nuclear localization in order to function in its characterized role of transcription induction, and the GFP-tagged Cln3 NLS screen displayed Cln3 NLS mislocalization in the absence of *NUP84*, we suggest that the absence of Nup84 protein hinders the nuclear localization of full-length Cln3. This same pattern in viability is observed in the *nup188* strain, which displays a relative decrease in its ability to form colonies when solely dependent on Cln3. Therefore, it is also possible that the Nup188 protein facilitates the nuclear localization of Cln3 (Figure 3). In contrast, assays utilizing the *nup2* strain did not display an observable difference in the ability of Cln3-dependent cells to form colonies relative to the viability of Cln2-dependent cells (Figure 3). The Cln3-dependent cells appeared equally viable regardless of the presence or absence of the Nup2 protein. Though Nup2 has been implicated as significant in the formation of chromatin boundaries during gene expression, there was no evidence in these viability assays that the absence of this nucleoporin interferes with the activity of Cln3.

We note that the spots on the experimental plate (YPD) of the *nup2* strain appear to have achieved more growth than the spots on the experimental plates of the *nup84* and *nup188* strains. If a phenotype that differs from the control is observed (such as was the case with the *nup84* and *nup188* strains), the plates are immediately removed from incubation and photographed. Following the experimental protocol, the

nup2 plates were observed for a maximum period of 48 hours, and therefore formed larger colonies, before they were removed from incubation and photographed.

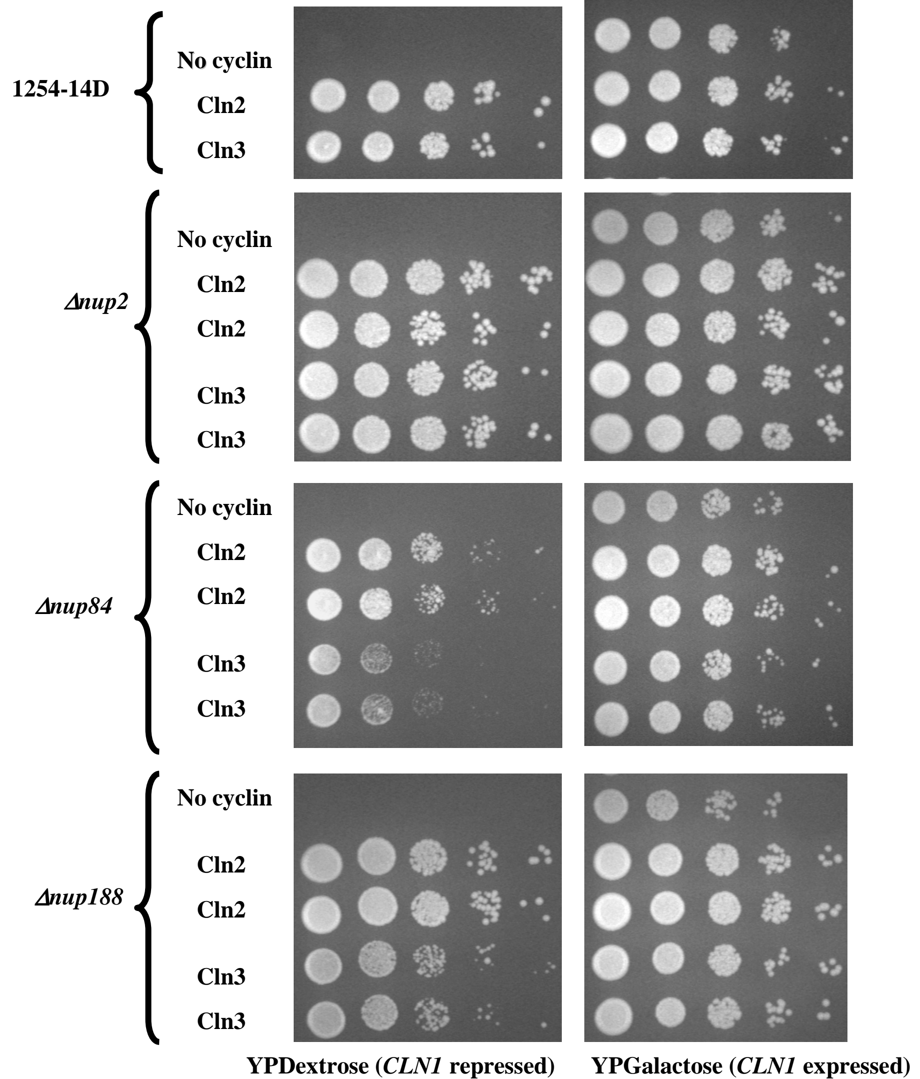

Figure 3: A series of ten-fold serial dilutions displays the ability of each strain to support colony growth in accordance with their dependence on Cln2 or Cln3. The top two photographs display the assay's wildtype controls, while the bottom six photographs show the viability of the *nup2, nup84*, and *nup188* deletion strain transformed with the specified plasmid. Cln2 and Cln3 are presented in doublets.The strains in each image are specified to the left of the figure, with Δ*nup2* signifying the strain MMY133-5A, Δ*nup84* signifying the strain MMY135-8D, and Δ*nup188* signifying the strain MMY131-12C. The carbon sources utilized are specified at the bottom of the figure. The plasmids used in strain transformations are indicated at the left of the photographs with No cyclin signifying pRS414, Cln2 signifying pMM82, and Cln3 signifying pMM99.

Though viability assay samples were incubated at both 30° C and 38° C, the relative defect in viability in Cln3-dependent nucleoporin-deletion strains was only observed at the elevated temperature. Since Cln3 is thought to respond to both intracellular and environmental stimuli (Morgan 2006), the elevated temperature may provide the additional cellular stress necessary to detect a viability defect. The network of proteins that interact with Cln3 is not well-characterized and additional research may resolve which proteins are affected by incubation at 38° C.

It is important to recognize that, though the viability assays in this research provide important insight into the reliance of full length Cln3 on the nucleoporins that were identified in the original genetic screen, they do not dismiss the possibility of proteins that function redundantly. For example, Bck2 has been implicated as having functions redundant with Cln3 but it was retained in the strain backgrounds for this investigation. If *BCK2* were deleted in these strains, it is plausible that the Cln3-dependent cells would display a stronger reduction in viability in the *nup84* and *nup188* strains. Similarly, the functions of the proteins that collectively comprise the nuclear pore complex have not been exhaustively characterized and it is possible that particular proteins are capable of rescuing the function of Nup2, Nup84, or Nup188 in their absence.

The second approach to establishing the relevance of Nup2, Nup84, and Nup188 to Cln3 activity examined the frequency of unbudded cells within a population. If Cln3 promotes passage through the Start checkpoint in the G1 phase, then Cln3 activity should share a negative correlation with the amount of time a cell spends in G1 (Cross et al. 2002, Morgan 2006). For example, a cell exhibiting decreased Cln3 activity should remain in the G1 phase for a longer period than a functional wildtype cell (Cross et al. 2002). In *S. cerevisiae*, cells develop buds at the beginning of S phase and retain their buds until division occurs in mitosis (Cross et al. 2002, Morgan

2006). Therefore, unbudded cells are considered to most likely be in G1 phase. Using this physiological trait, it is possible to estimate the frequency of cells that are in the G1 phase of the cell cycle in a culture of log phase cells (Zettel et al. 2003). If a strain possesses a defect in Cln3 activity, it should presumably display a higher frequency of unbudded (and thus G1) cells (Cross et al. 2002). Accordingly, comparison between the frequencies of unbudded cells was used to determine if the Cln3-dependent cells from any of the deletion strains displayed significant deviations from WT 1254-14D. Conducting budding indices aided in establishing the physiological relevance of any growth defects observed in the viability assay.

The wildtype *NUP* strain, 1254-14D, did not display a significant variation between the frequencies of Cln3-dependent unbudded cells relative to Cln2-dependent unbudded cells. These data support the idea that the two cyclins are equally capable of supporting progression through G1, and therefore budding at similar rates in the presence of the *NUP* genes. The Cln3-dependent and Cln2-dependent cells of the wildtype tester strain were observed to have an unbudded frequency of 27±2.4% and 25±5.7%, respectively (Figure 4). Accordingly, significant deviations from these control values in any of the nucleoporin deletion strains should provide insight into the relevance of these nucleoporins on the progression of the cell cycle through the Start checkpoint of the G1 phase.

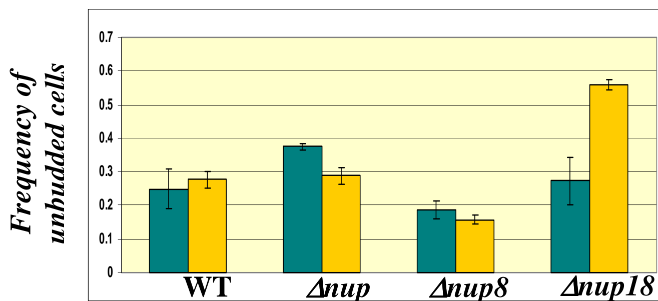

Figure 4: The Cln3-dependent *Δnup188* strain displays relative increase of unbudded cells. The frequency of unbudded Cln3-dependent cells (lightly-shaded columns) and the Cln2-dependent cells (darkly-shaded columns) in the wildtype *NUP* strain 1254-14D (WT), nup2 deletion MMY133-5A (nup2), nup84 deletion MMY135-8D (nup84), and nup188 deletion MMY131-2C (nup188) is shown. Error bars represent 2X standard error (calculated as [standard deviation x√3]x2).

Cln3-dependent deletion strains displayed aberrant morphology ranging from 26±4.5% (MMY131-12C) to 65±2.8% (MMY135-8D) of the population (Figure 5, 6), complicating the analysis of these data. Aberrant morphology was qualified by the presence of elongated processes, irregularly-shaped cells, and budding without complete cytokinesis (indicated by chains of buds that failed to disconnect), all of which prevented the accurate determination of the cells as budded or unbudded. Perhaps as a consequence of this elevated frequency of aberrant morphology, the frequency of unbudded cells in the Cln2-dependent or Cln3-dependent *nup84* strain was 16±1.3 % and 18±2.6 %, respectively. It may be misguided to analyze such data without first developing a strategy to qualify the presence or absence of a legitimate bud on Cln3-dependent cells.

Also worth noting is the apparently larger size of the Cln3-dependent cells in relation to the Cln2-dependent cells (Figure 5). If the typical function of Cln3 is considered, cells with defective Cln3 may take longer to pass through the Start checkpoint and out of G1 phase. In this case, cells that remain in G1 longer should be permitted more time in which to increase their size. Though cell size was not quantified during this research, the apparent increase in Cln3-dependent cell size may result from the inability for Cln3 to function in its wildtype capacity under the specified experimental conditions. The elevated temperature at which the cultures were incubated (38°C) may have acted as a stressor to induce the apparent increase in Cln3-dependent cell size regardless of the absence of Nup84, Nup188, or Nup2.

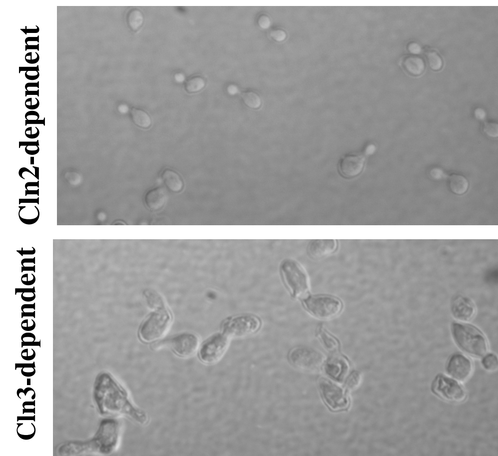

Figure 5: Cln3-dependent cells display morphology that is markedly different from Cln2-dependent cells. The images display cells with all of their nucleoporin-encoding genes intact (All cells in the figure are the 1254-14D wildtype *NUP* strain). The cells in the upper image are Cln2-dependent and the cells in the lower image are Cln3- dependent (signified to the left of each image). Prior to photography, cells were grown to log phase in YPD liquid media and fixed (see Budding Index section of Methods). All observations were made using an Olympus BX51 epifluorescence microscope in transmitted-light mode. Digital images were acquired with a SPOT RT-SE digital camera and its acquisition software (Diagnostics Instruments, Inc).

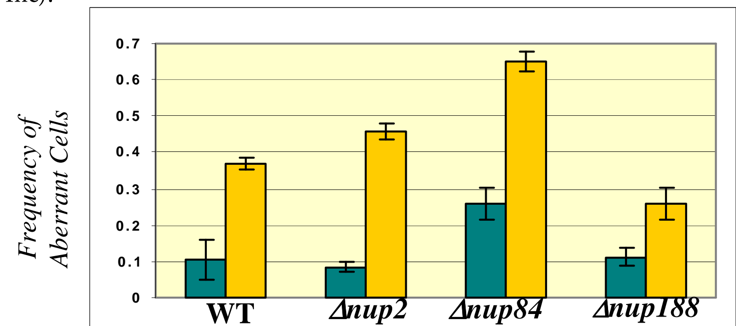

Figure 6: Cln3-dependent cells display a significant increase in the frequency of aberrant morphology. The frequency of

aberrant morphology Cln3-dependent cells (lightly-shaded columns) and the Cln2-dependent cells (darkly-shaded columns) in the wildtype *NUP* strain 1254-14D (WT), *nup2* deletion MMY133-5A (*nup2*), *nup84* deletion MMY135-8D (*nup84*), and *nup188* deletion MMY131-2C (*nup188*) is shown. Error bars represent 2X standard error (calculated as [standard deviation x√3]x2).

Of the three experimental strains, only samples from *nup188* displayed a higher frequency of unbudded Cln3-dependent cells (56±1.6%) relative to Cln2-dependent cells (27±7%). While the frequency of unbudded Cln2-dependent cells of the *nup188* strain is similar to the data obtained from unbudded Cln2-dependent cells of the wildtype tester strain, a difference of 29±2% separates the frequency of unbudded Cln3-dependent cells in the two strains. This data suggests that, in a log phase population of cells, the absence of Nup188 results in an approximately two-fold increase in the frequency of unbudded cells. Since Nup188 serves as a component of a subcomplex that comprises the core of the nuclear pore complex, it is plausible that Nup188 interacts with Cln3 to facilitate its transport through the length of the NPC (Figure 1).

The Cln2-dependent *nup84* strain displayed a frequency of unbudded cells (18.6±2.6%) that was statistically similar to the frequency observed in the Cln2-dependent 1254-14D strain (24.8±5.8). However, the frequency of Cln3-dependent unbudded cells in the *nup84* strain (15.7±1.2%) was statistically lower than the frequency of Cln3-dependent unbudded cells in the 1254-14D strain (27.6±2.4). The overall frequency of unbudded cells in the *nup84* strain was, at minimum, 7.0% lower than the frequency of unbudded cells in the 1254-14D strain (Figure 8). This trend seems to infer that cells from the *nup84* strain were progressing through G1 at a faster rate than the 1254-14D cells, but this may be a symptom of the high frequency of cells from the *nup84* strain that display aberrant morphology that was previously discussed.

Of the deletion strains researched, the data obtained from the cell characteristics of the *nup2* strain were shown to be most statistically similar with the results of the wildtype tester strain (1254-14D) (Figure 4). The frequency of Cln3-dependent unbudded cells diverged only 2±2.6% between the *nup2* strain and the 1254-14D strain. Similarly, the frequency of cells displaying aberrant morphology in the *nup2* strain and the 1254-14D strain was separated by 8±2.3%. These data are consistent with the idea that Cln3-dependent cells progress through the cell division cycle at a comparable rate regardless of the presence or absence of Nup2. Since commitment to cellular division is largely regulated by Cln3 activity, it may also be derived that Nup2 does not substantially affect the functionality of Cln3. These results agree with the viability assay of the *nup2* strain – both experiments would suggest that the Nup2 nucleoporin is not significant to the nuclear localization of full-length Cln3.

Concluding Remarks

Based on these data, it appears that Cln3-dependent cells are characterized by a viability defect in the absence of Nup188. Though not assayed directly, it might be inferred that the Nup188 protein allows the Cln3 cyclin to successfully navigate from the cytoplasm into the nucleus. In contrast, the research presents no data which suggests that Nup2 acts in a relevant physiological role to promote or alter Cln3 activity. While the results of the budding indices that incorporated the *nup84* strain do not appear to demonstrate a specific G1 defect, Nup84 may play at least a partially significant role in the proper activity of Cln3 (as observed in the viability assay).

The separate deletion of each nucleoporin-encoding gene appears to generate a unique impact on the viability of Cln3-dependent cells. This distinction is substantial because it refines the role of the NPC from serving as an inactive channel through which Cln3 is transported into a dynamic complex whose protein components interact with Cln3 differentially. In other words, simply removing a particular protein from the NPC does not disable the complex or result in a uniform alteration. It appears that, not only do Nup84, Nup2, and Nup188 perform different functions as components of the NPC, but they may also associate with Cln3 in three specific manners. These assays demonstrate that the approaches described are well established and likely to allow us to address the roles of additional nucleoporin components in Cln3 activity. Future research may better establish the current conclusions by investigating the possible effects of genes that are partially redundant with the investigated genes or gene products that are able to rescue the function of the proteins absent from the experimental strains. For example, Bck2 has been acknowledged to share a degree of function with Cln3, so developing a similar experimental strain background that is also *bck2* may strengthen the data collected on the interactions between Cln3 and the nucleoporins in this study.

The information collected will aid in determining the assemblage of proteins that Cln3 must interact with in its navigation from the cytoplasm to the nucleus. The regulatory activity of Cln3 is crucial to cell growth and division, so any insights gained in the functional requirements of Cln3 should benefit future research on *S. cerevisiae* as a model for the cell division cycle. For example, there are currently no publications that address the role of specific nucleoporins on the functionality of full-length Cln3. Though this research was not exhaustive in its exploration of nucleoporins that potentially interact with Cln3, the data provide information on a subset of such interactions that may be elaborated in future investigations. The data might also prove useful to the ongoing research concerning the involvement of the NPC in the regulation of gene expression and protein anchoring at the nuclear periphery.

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The Effect of Pregnancy Outcomes on Subsequent Mental Health

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The 1973 case Roe v. Wade, the Supreme Court ruled that the prohibition of abortion violated constitutional rights of privacy and legalized all first-trimester abortions. While over thirty years have passed since this landmark decision, abortion continues to be an issue of great debate among members of the medical community, religious institutions, legislators and the general public. One common objection to the procedure is the belief that abortion causes mental health problems. The past decade has been marked by numerous studies that provide evidence both for and against the claim that abortion is correlated with poor mental health. Interviews with women following their abortions reveal that most women are happy with their decision to have an abortion. However, based on the varying methods used for statistical analysis, longitudinal cohort studies report either that depression is more likely following an abortion than a delivery of an unintended pregnancy, or that women who choose to deliver are actually at a greater risk for depression. Some studies demonstrate that substance dependence, anxiety disorders, social phobias, and suicidal tendencies are more common in young women that abort unwanted pregnancies than in those that carry pregnancies to term. Additionally, the analysis of the admission rate of women to psychiatric institutions following pregnancy events indicates that more women are admitted following abortions than following deliveries. Nonetheless, further research is needed before any relationship between abortion and mental health can be decisively proven.

Introduction

Undoubtedly, one of the biggest issues at the forefront of current debates on women's reproductive rights is whether access to abortion should be legal or illegal. Since the United States Supreme Court's historical decision in the 1973 case *Roe v. Wade*, abortion has been a widely used procedure and a highly controversial topic in America. Each year in the US approximately 1.5 million legal abortions are performed (Major et al. 2000). On a more local note, in 2005 over 4,344 legal abortions were reported in Shelby County, while there were only 14,467 reported births (Division of Health Statistics, Tennessee Department of Health 2006). Objectively speaking, there are among legislators, religious officials, pro-life and pro-choice activists, and members of society at large, many religious, ethical, political, and medical reasons to support or to oppose such widespread legal access to abortion. One major point of opposition to abortion is the claim that abortion either directly or indirectly leads to psychological damage – including psychoses, depression, and post-abortion syndrome (PAS) - also called post-traumatic abortion syndrome or abortion trauma syndrome – an assortment of psychological and pathological characteristics thought to occur in some women following an elective abortion (Gomez and Zapata 2005). However, PAS is not recognized by either the medical community or the American Psychological Association (APA) as an actual syndrome. In fact, many believe that PAS is simply a fictitious syndrome invented by pro-life supporters and crisis pregnancy centers aimed at persuading women to choose birth over abortion. The actual existence of PAS and other abortion-related mental health issues continues to be debated among medical and psychiatric professionals, activists, abortion providers, and many others invested in the great abortion debate. In recent years, numerous efforts have been made to re-examine previous research results concerning mental health and abortion in an attempt to provide more evidence for both sides of the debate. Among the studies, results vary widely based on the sample set, controls, confounding factors, and the statistical methods employed. Nonetheless, widespread efforts have resulted in a handful of studies that individually support or refute the claim that women's mental health is dependent on pregnancy outcomes.

The lack of previous statistical support for either side of the debate on abortion and mental health is due in large part to methodological flaws or sampling biases in past studies. Post-abortion psychological issues have been either exaggerated by studies based solely on women who sought help for their problems or underrepresented in studies based on random samples of women requesting abortions (Major et al. 2000). The authors of recent studies also believe that earlier research has not investigated the psychological consequences of abortion over a long enough period of time. Previous studies may have evaluated abortion patients only as long as a few weeks or months after their procedure. Additionally, these studies often failed to specify whether the reported statistics on poor psychological health describe actual diagnosable disorders or simply an expressed regret or remorse on the part of the woman. Six studies on pregnancy outcomes and psychological responses have identified the shortcomings in previous research and attempted to provide more reliable statistics and conclusive results.

Discussion

Studying psychoses and registered mental illnesses can be a very fascinating research endeavor; however, to determine the effect of abortion on women, it is necessary to

first assess basic sentiments of remorse, regret, and guilt felt by women who have abortions. One study conducted in 2000 (Major et al. 2000) used a random sample of 442 women of reproductive age (with a mean of 24 years) arriving at 3 sites for a first-trimester vacuum-aspiration abortion to observe women's abortion-related emotions and general mental health. The sample included only women who reported that their pregnancy was neither intended nor the result of rape and was demographically similar to the national population in order to best represent the typical population of women seeking abortions. The 442 women constituting the final sample (those assessed at all stages of the study) filled out a questionnaire in the clinic prior to their abortion for a baseline (T1) assessment of their emotions regarding their pregnancy and decision to have an abortion. The other three assessments were conducted an hour after the procedure (T2), one month after the procedure (T3), and two years after the procedure (T4). Questions asked at T2 and T4 to determine abortion-specific emotional reactions required patients to rank six negative emotions, three positive emotions, and relief on a scale of one to five, with one meaning the emotion was not felt at all and five indicating it was experienced a great deal. Rankings for sadness, disappointment, guilt, feeling "blue", feeling "low" and feeling loss were averaged in order to determine overall negative feelings regarding the abortion; similarly rankings for happiness, feeling "pleased", and feeling satisfied were averaged to determine overall positive emotions regarding the abortion. With various other survey items women were asked to rank 1 to 5 whether or not they felt their abortion had a positive or negative effect on them ("appraisals"), if they were satisfied with their decision ("decision satisfaction"), and if they would make the same the decision to terminate their previous pregnancy over again ("do over"). Lastly, general mental health was evaluated at various times with the Brief Symptom Inventory for depression, the Rosenberg Self-Esteem Inventory and the diagnostic criteria for post-traumatic stress disorder outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM).

After responses were gathered, descriptive statistical methods were used to report responses for emotions, appraisals, decision satisfaction and do over as well as how these responses changed with time since the abortion. One hour after their abortion was performed, most women noted in the questionnaire that they felt more relief (mean reported 3.53 on a scale of 1 to 5) than any of the three positive emotions (mean 2.24) and more of the positive emotions than any of the six negative emotions (mean 1.90). However, these results did change some over time: the mean reported for relief two years after abortion decreased to 3.11, while the mean for positive emotions decreased to 2.06 and the mean for negative emotions increased to 2.16. For harm appraisals one month and two years after the baseline, most women felt their abortion did them more good than harm: the means reported for harm appraisal were 2.09 and 2.07 at T3 and T4 respectively while the mean benefit appraisals were slightly higher at 3.10 and 3.09. Results show that appraisals did not change significantly over time. Additionally at T3, 78.7% of women said that they were satisfied with their decision to abort while only 10.8% were dissatisfied and 10.5% were neutral. Although not drastically, decision satisfaction did change at T4, with only 72% reporting satisfaction and 16.3% reporting dissatisfaction. Similarly, two years post-abortion, 69% of women said they would make the same decision again if they had to, while 19% said they definitely would not and 12% were undecided. With regard to general mental health, although the mean reported depression score from the Brief-Symptom Inventory increased from T2 (.33) to T3 (.63) and from T3 (.63) to T4 (.72), the mean score was still lower at all times after the abortion than the pre-abortion mean (.86). Self-esteem also increased with time after the abortion - though it was still much higher post-abortion than pre-abortion - and only 1% of the sample qualified for post-traumatic stress disorder (PTSD).

This study concluded that "psychological distress after an abortion is rare" and that the "percent of women experiencing clinical depression within 2 years after abortion (20%) equals the rate of depression among all women 15 to 35 years of age (20%)" (Major et al. 2000). The rate of PTSD in the sample population was significantly smaller than the rates of PTSD in the population of women who have never had an abortion and the populations of female victims of rape or childhood physical abuse. Therefore, this 2000 study does not provide evidence to support either the claim that PAS is real or that mental health necessarily declines as a result of an elective abortion. The researches also found that the small percentage of those women who did demonstrate increased depression or lowered self esteem had several predictors of poor mental health post-abortion. Specifically, women who had a history of depression prior to their aborted pregnancy were more likely to become depressed after their abortion, and younger women and women with more children at the time of their abortion were most likely to negatively evaluate their abortion. Also, African American women showed a much higher overall self-esteem than women of other ethnicities.

Although the study mentioned above is important to the abortion debate because it may disprove the theory that abortion largely causes mental illness, there is another theory in support of access to abortion that holds that depression is independent of the outcome of a pregnancy. This suggests that a person is equally likely to become depressed whether they carry an unintended pregnancy to term or choose abortion. In 2002 David Reardon and Jesse Cougle published a study that evaluated this idea using data from the National Longitudinal Study of Youth (NLSY) that was initiated in 1979 (Reardon and Cougle 2002). Reardon and Cougle used the Center for Epidemiological Studies depression scale (CES-D) to gage depression in 6215 women who had either their first unintended delivery or abortion between 1980 and 1992. A CES-D score higher than 15 classified a woman as being at "high risk" for clinical depression. After logistic regression analyses, results showed that 27.3% of women who aborted their unintended pregnancy were at high risk of depression as compared with 22.7% of women who carried their unintended pregnancy to term. The mean depression scores for women who delivered and women who aborted were significantly close (8.88 and 9.09 respectively). Reardon and Cougle

determined that the difference in the percent of women demonstrating high risk for depression in each group was large enough to conclude that depression is in part dependent on the outcome of a pregnancy. This was the first study since the early 1990s to examine mental health in conjunction with both abortion *and* the delivery of an unintended pregnancy.

Despite the results of 2002's cohort research, Sarah Schmiege and Nancy Russo were dissatisfied with the study because of its pro-life bias – David Reardon is a pro-life activist and director of the online Elliot institute – and its inference that a *correlation* between abortion and depression proves that abortion *causes* mental health problems (Kranish 2005). Schmiege and Russo published their own article in 2005 with the objective of explaining the "discrepancies with previous research that used the same dataset" (Schmiege and Russo 2005). This study used the same data from the cohort study used by Reardon and Cougle, the NSLY. However it used coding approaches outlined by the staff of the NSLY to identify a smaller number of women eligible for the study – 1247 women – and did not exclude from the study women who had subsequent abortions from either the delivery or abortion group in order to remove bias. Using the same scale as the previous study (CES-D), Schmiege's and Russo's results generated similar depression scores for both the delivery and abortion groups. In fact, 28.6% of women who delivered their first unintended pregnancy were at high risk for clinical depression while only 24.8% of women who aborted were at high risk. The mean scores for the delivery and abortion groups were 11.8 and 10.8 respectively. Thus this study contradicts the findings of Reardon and Cougle and concludes that pregnancy outcomes do not predict depression.

Although the more recent study did not demonstrate a *direct* connection between exposure to abortion and depression, it did examine the *indirect* effects of abortion. Schmiege and Russo performed *t* tests comparing mean family income, education, and the number of children for the women in the delivery group and the women in the abortion group. These tests showed that women in the abortion group had a higher mean education and a higher mean income, as well as a lower family size. Specifically, families of women in the delivery group earned on average a combined income ranging from $20,000 to $30,000 while families of women in the abortion group earned a combined income on average between $30,000 and $40,000. Additionally, women in the delivery group had on average little more than a high school education, while women who aborted had completed more years of upper level education. Thus, the authors of this study reported that "although there is no credible evidence that choosing to terminate an unwanted first pregnancy puts women at higher risk of subsequent depression than does choosing to deliver an unwanted first pregnancy . . . delivering a first unwanted pregnancy is, however, associated with lower education and income and larger family size – all risk factors for depression" (Schmiege and Russo 2005). This outcome is consistent with other reported information on the negative effects of unwanted early childbearing.

Despite their conclusions, the three studies mentioned above may have been flawed due to a failure to completely control for pre-pregnancy mental illness – which certainly skews statistical results. A fourth study, published in 2007, sought to determine – after adjusting for a previous history of depression - whether or not abortion is associated with a *different* risk of major depression (MD) than the risk before the pregnancy (Rees and Sabia 2007). In order to do accomplish this, the researchers obtained a sample that could be controlled by taking advantage of a newer national longitudinal study not already used in abortion-depression research called the "Fragile Families and Child Wellbeing Study". The sample included 2844 new mothers living in 20 highly populated cities and was significantly representative of the wider population of mothers in metropolitan areas. For the study, baseline assessments of depression were made in hospitals following births between 1998 and 2000. Subsequent interviews were conducted one year and three years following the first assessment. Rees and Sabia used the Composite International Diagnostic Interview Short Form (CDI-SF) from the DSM to determine mental disorders in the new mothers. Women were assigned a "major depression (MD)" score based on the number of depressive symptoms they exhibited for a period of longer than two weeks – feeling sad, blue, or depressed, losing interest in most things, feeling tired, weight changes, trouble sleeping, trouble concentrating, feeling down and thinking about death. This study can be compared with previous studies because it also categorized women with three or more symptoms as being at high risk of major depression. The risks (or symptoms) of clinical depression were correlated with 15 categories of pregnancy outcomes at subsequent follow ups, the three most important for this study being "birth between follow-ups", "abortion at follow-ups", and "did not become pregnant between follow-ups".

Statistical analysis for the Fragile Families study indicated that at the first and second follow-ups, 20.3% and 31.6% of women with a reported abortion subsequent to the baseline assessment were at risk for clinical/major depression. Of the women who gave birth subsequent to the baseline, 14.0% and 21.0% were at high risk for MD at the first and second follow-ups, respectively. Meanwhile, 13.0% and 15.5% of women who did not become pregnant between follow-ups were at risk. Subsequently, a multivariate logistic model was made to adjust for personal and household characteristics that could confound the results. Still, abortion was positively correlated with a more than two-fold increase in symptoms of MD three years after the baseline assessment. Interestingly, giving birth between follow-ups was still associated with a higher risk of MD at the second follow up compared with women who did not become pregnant. The results seem to agree with Reardon and Cougle's conclusions; however their reliability is contested because of a few methodological flaws. Among these flaws are that the results can only be generalized for the population of new mothers in large urban US cities. Additionally, the study did not ask participants whether or not their pregnancies were intended or unintended, wanted or unwanted. Furthermore, although the research does show a positive correlation between abortion and depressive symptomatology, results also report that giving

birth is correlated with a similar risk. For this reason, rather than agreeing with Reardon and Cougle, the researchers of the Fragile Family study state in their conclusions that their research result is "similar to that found by Schmiege and Russo, who examined unwanted first pregnancies among young women" (Rees and Sabia 2007).

While there have been several recent studies examining depression as a factor of pregnancy outcome, a study conducted in New Zealand in response to publications by Reardon and his critics investigated additional mental health responses to abortion such as anxiety, suicidal behavior, and substance dependence and abuse in a younger sample of girls and women (Fergusson et al. 2006). The research team recognized that although some research points to the theory that exposure to abortion increases the risk of depression, the contrapositive suggests that pregnancy without abortion is beneficial for mental health. This study attempted to study mental disorders beyond those addressed in previous studies and to compare young females who aborted unintended pregnancies, delivered them, or never became pregnant at all. Like preceding studies, this team used data from a longitudinal cohort study of children in urban New Zealand from birth to 25 years. From the 1265 children in the study, there was a sample of 506 females for whom there were both complete mental and pregnancy histories. These 506 females were interviewed at 15, 16, 18, 21, and 25 years of age. At each assessment, pregnancy and birth histories were obtained relative to the last interview and the participant was evaluated – according to DSM criteria - for depression, anxiety disorders, agoraphobia, social phobia, alcohol dependence, illicit drug dependence, and suicidal behavior. Adjustments were made for covariate factors that would confound the results such as family socio-demographic background, family functioning, problematic childhood conduct, education level reached, childhood personality, and young-adult living arrangements.

Results indicated that every mental health issue except alcohol dependence – illicit drug dependence, total mental health problems, suicidal ideation, and depression – was significantly associated with a history of abortion. Even anxiety disorders were marginally associated. Pooled risk ratios show that females who became pregnant but did not have an abortion were 24% as likely to have suicidal thoughts as females in this age group who aborted pregnancies – those who did not become pregnant were 42% as likely. The "pregnant no abortion" group was only 15% as likely to be dependent on illicit drugs while the "not pregnant" group was 20% as likely as the "abortion" group. The "not pregnant" group was 66% as likely as the "abortion" group to develop mental health problems overall, while the "pregnant no abortion" group was only 58% as likely. The conclusions from this study are similar to Reardon's in that they provide evidence that exposure to abortion increases the risk of subsequent mental health problems. However, this study surpasses Reardon and Cougle's in its examination of disorders other than depression that may also be correlated with abortion. Interestingly enough, the research team involved in this study continued the discussion of its results with an appeal to pro-choice supporters – "exposure to abortion is a traumatic life event which increases longer-term susceptibility to common mental disorders" – and a blatant criticism of the APA's 2005 statement that PAS is not real and that psychological harm in response to abortion is low (Fergusson et al. 2006).

A sixth and final study provides adequate comparison with the numerous cohort studies based on interviews and self-assessments. This study, conducted once again by Reardon and his associates, deviated from previous research in several ways: it was entirely record based (it utilized records of the California Department of Health Services (DHS)), it controlled for socioeconomic factors by using a sample of low-income women all under the government-funded medical insurance program Medi-Cal, and it examined psychiatric admissions rates rather than assessed symptoms (Reardon et al. 2003). This allowed for a large sample size: 15,299 women whose first known pregnancy ended in abortion and 41,442 women whose first known pregnancy ended in delivery and had no subsequent abortions. Psychiatric admission was determined by extracting procedure codes for inpatient psychiatric claims (ICD-9) from records. Logistic regression analyses were then used to determine psychiatric admissions for the time periods 0-90 days, 90 to 180 days, and 0 days to 1 year after abortions or births as well as the admissions for the second, third, and fourth years after the pregnancy event. Statistics were adjusted to allow for confounding factors and stratified into age ranges.

The record-based study showed that 434 different women were admitted at least one time for psychiatric treatment during the four years after a pregnancy event (abortion or delivery). When the admission rate was adjusted for every 100,000 women per year, data showed that significantly more women per 100,000 from the abortion group were admitted than women from the delivery group for each of the six time periods under study. The difference in admission rates is surprisingly most drastic for the time period of 90 days following the pregnancy event: 408.4/100,000 women in the abortion group were admitted as compared with 152.5/100,000 from the delivery group for this time period. The authors of the study found this counterintuitive because one would anticipate the reverse – with admissions from the delivery group at this time being expectedly high due to post-partum depression and admissions from the abortion group being low due to relief soon after the abortion procedure. The increased admission rate for the abortion group is discussed by the researchers and attributed in part to the fact that there is often less social support for women who abort than for women who carry their pregnancies to term and deliver. With regard to age, admission rates for the abortion group were significantly higher than rates for delivery groups for each of the five age ranges, but the most obvious rate differences occurred for the ranges from 13-19, 20-24, and 35-49 years of age.

This study also examines in detail the specific psychological disorders for which the women in the sample groups were admitted. These include depressive psychoses (single and recurrent episodes), bipolar disorder, neurotic ,

nonorganic psychoses, schizophrenic disorders, and adjustment reactions – a short-term psychological disturbance marked by depression, anxiety or sleep disorder-like symptoms in response to a stressor, in this case a pregnancy event (Hales et al. 2007). Researchers concluded that women who had abortions had significantly higher admission rates in the categories of adjustment reactions, single and recurrent depressive psychoses and bipolar disorder and that the number one diagnosis for all women was single-episode depressive psychosis. Reardon's second study also supports, not surprisingly, the belief that abortion is correlated with psychological damage. However this study may be somewhat flawed in that access to medical histories does not account for psychiatric admissions or pregnancy events for more than a year prior to the examination period.

Although the studies on abortion and mental health discussed above mark significant improvements over the studies from preceding decades, the results and conclusions of each are clearly not in agreement. In conjunction, they do little to accurately settle the debate on the existence of post-abortion syndrome and related mental health outcomes. However, there is one common conclusion noted by each research team – there is an obvious need for better, more long-term studies in order to resolve the continuing controversy. It remains clear that one of the best ways to deal with poor mental health related to all pregnancy outcomes of unintended or unwanted pregnancies is to prevent unintended or unwanted pregnancies. If the church, state, medical and psychiatric professional communities, society at large, and activists were truly concerned with reducing women's risks of depression and poor mental health, they would focus on the prevention of unplanned pregnancies - and thereby the reduction of possibly damaging incidences of abortion and unwanted deliveries - rather than on narrowly focused, methodologically flawed research on the risks of a procedure supported by thirty-five years of Supreme Court approval.

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Arboreal Biogeography: The Effects of Physical Gradients on the Size and Spatial Distribution of *Ocotea usambarensis* Mazumbai Forest Reserve, Northern Tanzania

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The evergreen tree Ocotea usambarensis (family Lauraceae) was surveyed for abundance and size over 18 days in November 2007 in the Mazumbai Forest Reserve in Northern Tanzania. These data allowed me to determine the effects of geographic heterogeneity at macro and micro-spatial scales on arboreal size and distribution. An altitudinal preference was evident at the macro-scale. At the micro-scale, local topography significantly influenced size, while both undulation shape and slope were significantly correlated with abundance, implicating a geographic determinant at this scale on arboreal size and distribution. The entire sampled population was found to be clumped, a result of the combined effects of microsite selection and seed predation. This study indicates the capacity of purely geographic factors to maintain non-uniformity in the forest through the creation of species-specific preferred microsites. This mechanism of spatial heterogeneity acting in multiple scales contributes to diversity of species.

Introduction

Background

The rainforests of East Africa are highly fragmented islands of vegetation with localized rainfall, surrounded by areas of much more arid woodland (Wasser and Lovett 1993). These fragmented patches are the remnants of the pan-African forest. Before the breakup of Gondwanaland, a supercontinent consisting of present day Africa, India, Madagascar, South America, Australia and Antarctica, during the Karroo period (300 myr bp), the Eastern Arc Mountains were uplifted by block faulting and volcanic activities (Griffiths 1993). This rifting develops when the continental plate is stationary over rising mantle, resulting in geological swells interspersed with basins (eg. Lake Victoria, a basin lake located between the east and west arms of the Rift Valley). 100 myr bp, Gondwanaland began to break apart, drastically changing the geological and climatic conditions of the region.

At this time, Africa was 15-18 degrees south of its present position with the equator running though what is now the Sahara (Lovett 1993). Before the fragmentation and later complete breakup of Gondwanaland, East Africa experienced an arid climate, due to the fact that the landmasses later to become Madagascar and India were situated between what is now East Africa and the nearest body of water, the Tethys Sea (Griffiths 1993). As the supercontinent began to fragment into its respective landmasses, the newly formed East African coast began to receive increased amounts of moisture from the Tethys Sea, later to become the Indian Ocean (Lovett 1993). Africa also began to drift north towards its present day position. Equatorial rainfall is created by "oceanic solar heating" via the intertropical convergence (ITCZ) zone (Lovett 1993).

Rainfall, and subsequently, forest growth, is dependent on the position relative to the equator (Lovett 1993). During this timeframe, the area experienced forest growth due to its new climate and position relative to both the equator and ITCZ. About 7 myr bp, the continued uplift of the Eastern Arc and Nyasa Rift Mountains created a rainfall gradient, thus fragmenting the rainforest into local islands. As a result, the side of the range facing the east continued to experience high rainfall while the west side suffered from a rain shadow and received relatively little rainfall (Lovett 1993).

The Usambaras Mountains, located in the Eastern Arc range (Appendix A), contain rainforest fragments that are unique in East Africa for their high species richness and diversity and are recognized as being within the top 24 top biodiversity hot spots in the world. Within this range, 2100 species of vascular plants have been identified with a level of endemism estimated at 25-39% (Pantaleo 2001). The isolation and fragmented nature, combined with the sheer age of these rainforest remnants allows for this high number of endemic species. These forests are at least 30 million years old, and they have been isolated from west and central African forest for at least the past 10 million years (Lovett 1998). Paleoendemics found here are left over from the former distribution of the pan-African rainforest and the neoendemics have emigrated from surrounding habitats and have had enough time to adapt to the forest due to its age (Pantaleo 2001). The forests were possibly utilized as a refuge for species from other tropical forests during the era of Pleistocene climatic changes, taking place in the last 2.3 million years (Lovett 1998).

Worldwide, a “tropical gradient” is evident. Many taxonomic groups experience a substantial increase in

diversity with the latitude shift from temperate zones to topical zones, with trees manifesting one of the greatest differences. Tropical forests are also far more structurally complex than temperate forests, a factor that helps to accommodate the increase in species diversity. These forests are highly productive ecosystems that exceed other types of vegetation in terms of sheer photosynthetic activity (Terborgh 1992). This productivity, however, is a bit of a paradox. The mineral soil found beneath many tropical forests is almost barren of soluble minerals, the result of nutrient leaching caused by eons of torrential downpours. The nutrients that make tropical forests so astounding in stature lie in both living and recently dead organic litter (Terborgh 1992). These plants recapture dissolved nutrients during their release at decomposition, with the mineral soil only serving as a water source and anchor point. Due to this strong nutrient recycling and high nutrient capture and retention rates, there is no clear correlation between forest biomass and soil fertility (Terborgh 1992). Competition between species exists for soils with certain ranges of nutrient levels, fueling the flora diversity inherent to tropical forests (Terborgh 1992).

Soil and nutrient availability vary spatially in tropical forests on several different scales, particularly at a micro level. This heterogeneity extends on to encompass the physical landscape. Geographic factors, such as altitude, hydrology, aspect, and topography, all act to drive the distribution and diversity of the vegetation community structure. The effects produced by such physical factors are much more drastic on mountain systems, where geographic gradients can change abruptly (Pantaleo 2001).

Altitude is a particularly influential factor in the distribution and condition of vegetation. Altitudinal zonation of tropical forests is a widely accepted and common practice; however, studies have shown vegetation communities to be both continuous and discontinuous with respect to altitude (Pantale 2001). Altitude is related to climatic pressures and as a general rule altitude increases while temperature decreases. Nevertheless, a static relationship is not evident between the two. The rate of temperature change is affected by cloud cover, region, seasonality, rainfall, condensation, and humidity (Richards 1996). In Pantaleo's (2001) study of the region, the strongest correlation with community composition was elevation.

Topography is another major factor of vegetation distribution. Small-scale topography influences vegetation by dictating the availability of water. Thus, a topographically concave area with the water table physically closer to the surface may experience waterlogged soil and soil oxygen diffusion may be reduced as a result, possibly leading to roots dying from anoxia (Terborgh 1992). In a convex area, where the water table is relatively deeper, moisture stress may be experienced. Heterogeneity in the tropical forest works on both macro and micro scales, fueling overall flora diversity and distribution. The result of the environmental and geographic variables is a vegetation mosaic that is highly stratified, with extreme variation in productivity.

In this study, I will be examining the size and distribution of *Ocotea usambarensis* in respect to topography (undulation shape and slope) and altitude. *O. usambarensis* (also known as East African camphor) is a pioneer species generally found at altitudes above 1000 meters above sea level (masl); its primary range being East Africa with adjacent areas in Zambia, Malawi and DRC (Holmes 1995; Schulman et.al. 1998). This species was selected because it is found at all elevation zones of the study site and can therefore serve as a accurate indicator of the heterogeneity of the growing conditions as dictated by geographic factors. This study also hopes to provide some insight on the biogeography of the species itself. It also is one of the most abundant species of tree in the Usambaras range and makes up a significant portion of the basal area (Pantaleo 2001).

I suspect to see an increase in abundance of *O. usambarensis* with altitude (personal observation; personal communication. Mrecha) and an increase of density in concave topographic areas. As for DBH, I expect to see a decrease with altitude due to increased environmental stress and an increase of DBH in locally concave areas. I anticipate that area with extreme degrees of slope will harbor substantially less individuals then areas with moderate degrees of slope. I also expect to see a relatively clumped spatial distribution rather than evenness due to microsite selecting.

Study Site

Rainforests in East Africa consist of 10,000km^2, making up only 0.1% of the total world rainforest area (Lovett and Wasser 1993). Despite their relatively small total area, the montane forests of East Africa are recognized as a biodiversity hot spot with high levels of endemism (Pantaleo 2001). These highly fragmented remnants of the pan-African rainforest still exist primarily due to the stability of the Indian Ocean currents to provide moisture (Lovett and Wasswe 1993). As of a result of this fragmentation, these montane forests consist of pockets of localized rainfall surrounded by relatively more arid woodlands. This isolation from the main forest blocks found in West Africa is a huge contributing factor to the species diversity (Iddi 1998). More than half of these forests are found in the crystalline Eastern Arc Mountains, running from southern Kenya to southern Tanzania (See Appendix A). The Eastern Arc Mountains are the oldest mountains in east Africa and while making up only 2% of the total land area of Tanzania, these forests harbor more then 40% of the country's natural flora and fauna (Idii 1998). These remaining islands of forest (sometimes known as the "Galapagos of Africa") are preserved for the most part due to their contributions as watersheds for the surrounding communities (Redhead 1981, Iddi 1998). The Eastern Arc Mountains act as the major catchment for most of the larger rivers within Tanzania. The Usambaras, contained within the Eastern Arc Mountain Range, serve as prime example of the island biogeography and fragmentation typical of East Africa rainforests. They consist of seventeen patches of vegetation, making up a total area of 328 km^2 (Newmark 1998). The Usambaras in particular also feed into the Pangani River, which supplies water to a large number of coastal communities (Iddi 1998).

The Mazumbai Forest Reserve (See Appendix B) is one of the only accessible East African forests still existing in its primeval state (Redhead 1981). The Reserve is located on

the eastern face of the West Usambaras, is a protected area of low-elevation montane rain forest. The reserve contains 320 hectares and is bordered on nine kilometers of its fifteen kilometer perimeter are bordered by hard-edge deforested areas. Baga I and Baga II, government-owned forests, also border the western and southern sides (Cohen 2006). It lies at 4 50'S, 38 30'E. Footpaths run through the forest and the Reserve is guarded full time to prevent the locals from harvesting firewood (personal observation; personal communication, Mrecha). The location of the Reserve causes it to receive 1230mm of rain annually on average, making it one of the wettest areas in the range (Pantaleo 2001; Redhead 1981).

Five distinct vegetation communities have been distinguished on the basis of altitude. 1360-1650 masl is characterized by: *Sorindeia usambarensis, Parini exelsa, Newtonia buchanani,* and *Allanblackia stuhlmanii.* 1390-1840 masl-*Strombosia scheffleri, Cradibea brevicaudata, Pachystela msolo,* and *Isoberlinia scheffleri*. 1415-1800 masl-*Syzygium guineense, Sorindeia usmbarensis, Parini exelsa,* and *Newtonia buchanani.* 1430-1880 masl-*Ocotea usambarensis, Syzgium guineense,* and *Parini exelsa*. 1570-1910 masl-*Agauria salicifolia, Ocotea usambarensis, Cryptocaria liebertina,* and *Aphloia theiformis* (Pantaleo 2001).

The Reserve was first owned by the Swiss farmer, John Tanner, who planted tea. Ownership was transferred to the University of Dar es Salaam in 1968 to operate as a research forest. Management was later assumed by the Sokoine University of Agriculture. Tanner's Swiss chalet is located approximately 750 meters outside of the Reserve, near the road that neatly bisects the forest at 1500 meters above sea level (masl) (Cohen 2006; Redhead 1981).

Methods

Collection

This study was conducted in the Mazumbai Forest Reserve from the 10th-27th of November 2007, during the short rains. The sample frame was the whole of the Reserve, with *Ocotea usambarensis* being the sampled population. Nine hectares were sampled in six altitudinal ranges: <1400m, 1400-1500m, 1500-1600m, 1600-1700m, 1700-1800m, and1800-1900m, for a total surveyed area of fifty-four hectares--making up seventeen percent of the sample frame. This constitutes a representative sample. Plots of one hectare were systematically distributed within each altitudinal zone, arrayed from south to north, along the topographic gradient in order for altitude to remain relatively constant. Three days were spent surveying each altitudinal zone, with three hectares sampled per day. Plots were spaced at 50, 100, 150, or 200 meter intervals, in order to be proportionately spaced in respect to the total area available in each zone. This practice was necessary because the available area at each altitude range varied significantly (see Appendix B). Systematic placing of the plots was necessary to ensure that sampling was conducted randomly with respect to the surrounding environment. A one hundred meter buffer zone was maintained with the edges of the Reserve, as this is thought to be the maximum distance of edge effects (personal communication, Mrecha). Sheer drops and areas with a slope substantially above sixty degrees were considered impassable terrain for sampling purposes and were thus skirted to preserve my mental heath.

When a plot's location was determined, a fifty meter centerline was laid along the contour line and an additional fifty meter line was laid perpendicular to the centerline on both uphill and downhill sides. This procedure was repeated for the opposite side of the plot. In this manner, the plot, which enclosed a single hectare (100m by 100m), was split into more manageable quarter-hectare (50m by 50m) quadrats for ease of sampling and layout. This also allowed for a higher level of resolution when the sampled population was tested for clumping.

Topography and slope were then collected on a quadrat scale. Topography and slope were too heterogeneous to be quantified on a hectare scale. Topography was characterized as concave, intermediate, and convex, determined observationally. Slope, also determined visually, was categorized by approximate degrees: 15, 30, 45, or 60. Metadata included whether or not quadats were in proximity (fifty meters or less) to a forest gap.

Individuals of *Ocotea usambarensis* with a DBH (diameter at breast height) above thirirty cm were counted and measured. DBH was taken on the uphill side of the individual and if buttressing was present, DBH measurements were taken above it. A tape was used to measure circumference, which was later converted to diameter.

Altitude was known for the bottom and top edges of the Reserve and for the bisecting road. With this data, my forester and I were able to ascertain the ranges of the six altitudinal zones with approximately one hundred meters of elevation change per
zone.

Analysis

Regressions were run against abundance and altitude and against DBH and altitude. Three-Way ANOVAs tested the significance of topography, slope, and altitude (individually and interactively) on both DBH and abundance. Tukey post-hocs were done as a follow-up. Distribution was determined by testing the goodness of fit of the Poisson distribution by chi-square analysis.

Results

For the macro-scale analysis of the effects of altitude on density and DBH, two regressions were run. Neither yielded significant results. The trendlines show extremely poor fits, implicating that the changes in either density or size cannot be attributed to a linear relationship with altitude across all sampled zones (Figure 1 and 2).

Three-Way ANOVAs were run to test the effects of topography, slope, and altitude, when independently considered and while acting in concert, on density and size (see Appendix D for all ANOVA p-values). On a micro-scale between altitudinal zones, altitude was found to be an influential factor on distribution with altitudinal preference shown for 1500-1600 masl (Figure 3.) Altitude also significantly affected size, with the largest average DBH being found in the 1400-1500 masl zone (Figure 4).

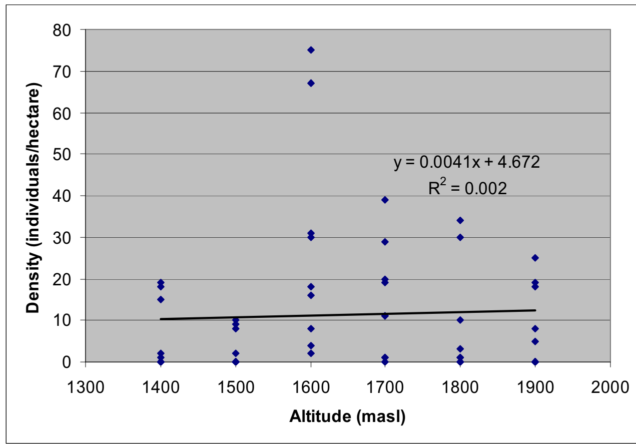

Figure 1: The Number of *O. usambarensis* Individuals Found in 54 Plots Across six Altitudinal Zones, Mazumbai Forest Reserve, n=620, Northern Tanzania, November 2007.

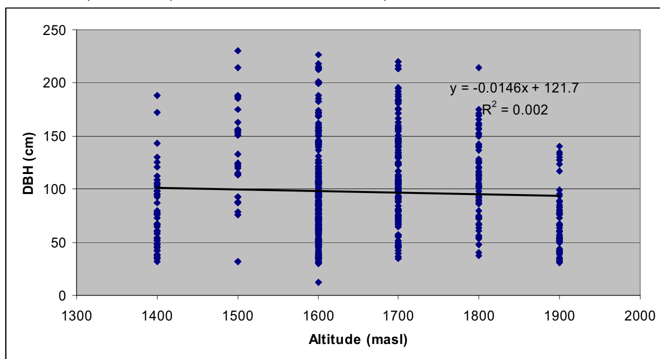

Figure 2: The DBH of *O. usambarensis* Individuals Found in 54 Plots Across Six Altitudinal Zones, n=620, Mazumbai Forest Reserve, Northern Tanzania, November 2007.

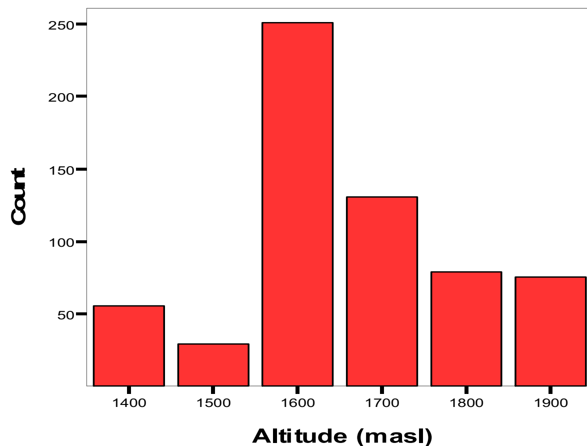

Figure 3: Number of *O. usambarensis* Individuals found per Altitude Zone, n = 620, Mazumbai Forest Reserve, Northern Tanzania, November 2007. Bars show counts.

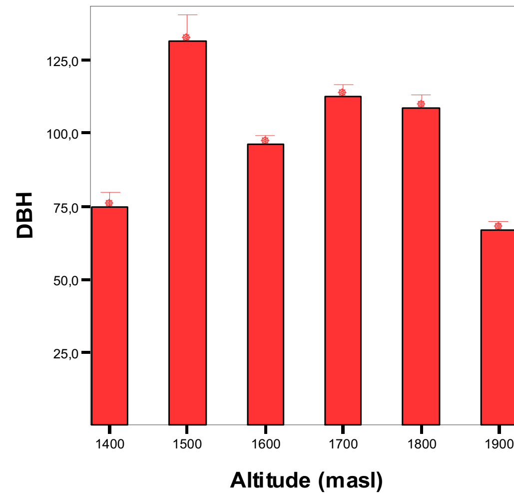

Figure 4: Average DBH of *O. usambarensis* Across Altitudinal Zones, Mazumbai Forest Reserve, Northern Tanzania, November. Bars show means. Error bars show mean ± 1.0 SE.

Topography type was shown to be a significant influence on the number of individuals found per quadrat, with the relative abundance of *O. usambarensis* individuals being highest in convex areas (Figure 5; Appendix D for P-values). Topography type also significantly affected size with the largest average DBH being found in concave areas (Figure 6).

Slope significantly influenced the number of individuals present, with an intermediate slope being the preferred area (Figure 7). Slope was not found to significantly affect size.

Topography and altitude were found to have an interactive effect, significantly influencing size (Table 1). The number of individuals found per quadrat was significantly affected by this interaction was well; however, the interaction of all three independent variables did not significantly affect abundance, despite the significance of these variables when acting independently (Table 1).

For macro-level distribution across all altitude zones, a Poisson distribution was tested for goodness of fit (Figure 8). For obtaining the expected values, the Poisson probability formula was used (See Appendix E for complete chi-square table and relevant formulas). The resulting chi-square yielded a significant p-value (<.000), giving strong evidence of a clumped population.

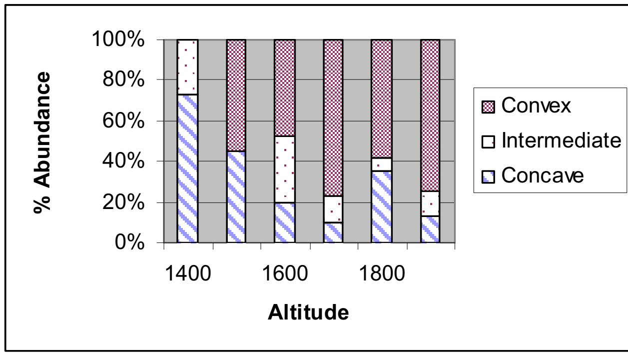

Figure 5: Relative Abundances of *O. usambarensis* Found in Three Topography Types Across Six Altitudinal Zones, Mazumbai Forest Reserve, Northern Tanzania, November 2007.

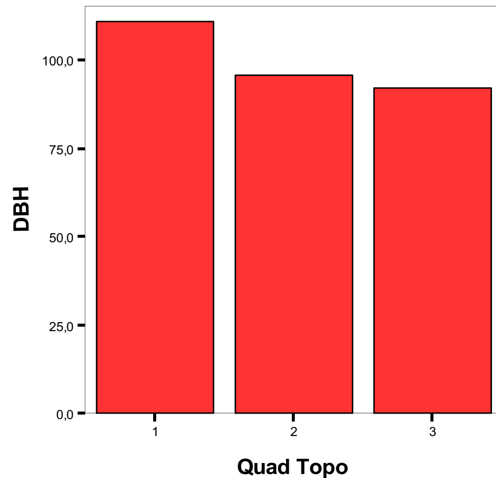

Figure 6: Average DBH of *O. usambarensis* Across Local Topography, Mazumbai Forest Reserve, Northern Tanzania, November 2007. Bars show mean.

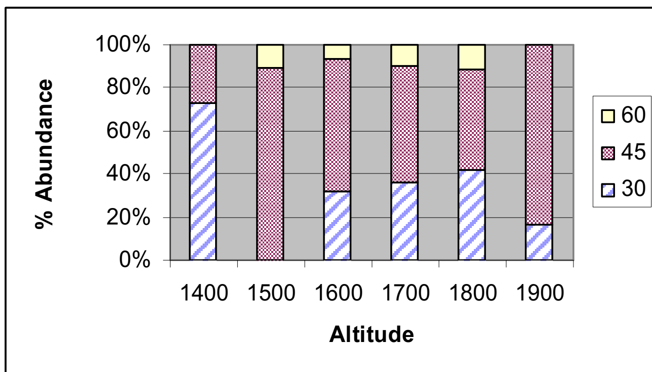

Figure 7: Relative Percentages of Slope Across Six Altitudinal Zones, Mazumbai Forest Reserve, Northern Tanzania, November 2007.

DBH, ANOVA	P-Value
Topography*Altitude	.001
Number of Individuals, ANOVA	
Topography*Altitude	.022
Topography*Altitude*Slope	.370

Table 1: Interactive Effects of Micro-Scale Variables on DBH and Number of Individuals Found. Interactive variables affecting size and distribution of *O. usambarensis*, Mazumbai Forest Reserve, Northern Tanzania, November 2007.

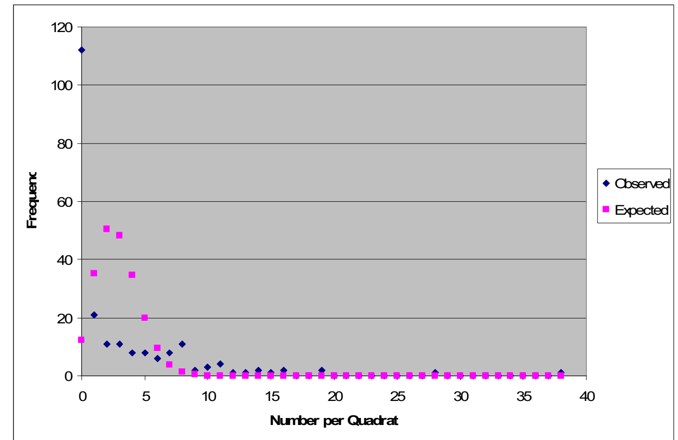

Figure 8: Poisson Distribution of Frequency of *O. usambarensis* Individuals per Quadrat, Series 1 Indicates Observed, Series 2 Indicates Expected.

Discussion

Contrary to my initial predictions that altitude would cause an increase of abundance and a decrease in size, the macro-level variable of altitude was found to not significantly affect either *O. usambarensis*'s DBH or the number of individuals found per hectare (Figure 1 and 2). This lack of a linear trend across altitude zones suggests that the relationship between the size and distribution of this species and large-scale physical gradients may be more complicated than previously imagined. However, clarification lies with the micro-scale variables.

While linear regressions showed altitude to be insignificant, it should not be discounted as an influential factor. The ANOVA run against altitude on a micro-scale (between altitudinal zones, rather than across) and the number of individuals found per quadrat showed significance (Appendix E for p-values). Although a visible trend across all altitude zones is not evident, *O. usambarensis* is shown to have an altitudinal preference, or at least a zone that best fosters its occurrence (Figure 3). 251 out of 620 individuals were found in the 1500-1600 masl range, far more than were found at any other altitudinal range. Temperature preference is a likely candidate for the explanation of this. Individuals of *O. usambarensis* in Amani Nature Reserve, also within the

Eastern Arc Mountains, have been almost unable to reproduce below 1500 masl due to changing climactic conditions, showing the sensitivity of this species to temperature (Schulman et.al. 1998).

DBH was also found to be significantly affected by altitude on a micro-scale. Individuals of *O. usambarensis* with the largest DBH were found in the 1400-1500 masl (Figure 4). If a preferable altitude range is evident, then it would be logical to assume that both the number of individuals found and the average DBH would be highest in this preferred zone. It is possible that this study sampled at a level of altitudinal resolution (100 vertical meters per zone) too high for these ranges to coincide with each other. The other possibility is that the level of altitudinal resolution is appropriate and growth is best fostered at a slightly lower altitude due to possible soil gradients not sampled in this study.

Topography proved to be a significant influence on individual abundance (Appendix E for p-values). Small-scale topography's influence is likely due to its inherent connection with the water table. Concave areas are locally closer to ground water sources and are much more susceptible to run-off than intermediate and convex areas. The prediction that the highest abundances would occur in topographic concavities was shown to be false. The relative abundances of *O. usambarensis* were found to be highest in topographically convex areas (Figure 5). Water availability is an apt explanation of this selection force. It is possible that *O. usambarensis* does not possess a root system capable of anchoring itself in the waterlogged soil that would exist in a concavity in the rainforest. This evidence would also suggest that the species may have a low tolerance for the decreased oxygen diffusion that would accompany waterlogged soil. It is also possible that concave areas may be more susceptible to nutrient leaching than other areas due to the increased water flow caused by runoff. In addition, most concave areas, especially in this lower altitude, were found to contain streams (personal observation, Long).

Topography significantly affected DBH as well, showing my earlier prediction to be correct (Appendix E for p-values). However, the opposite trend was seen. Individuals of *O. usambarensis* with the highest average DBH were found in topographically concave areas (Figure 6). This shows that individuals who are able to structurally support themselves in concavities are able to take advantage of the increased proximity to the water table. This, coupled with the fact that topography significantly affects abundance in the opposite direction, lends itself to the conclusion that *O. usambarensis* has a topographic preference due to structural concerns rather than nutritional ones.

While slope was not an influential factor of size, it did affect the number of individuals found per quadrat (Appendix D for p-values). This shows slope to be less important as a growing condition than as a factor of original colonization. Slope is a determining factor of whether or not a given area contains individuals, manifesting a habitat preference but not a major player in size. As predicted, relative abundance was highest in areas of intermediate slope (Figure 7). This is likely contributable to the anchoring and structural support systems in the species. Perhaps this is a weakness of the root system-to not be able to cope with the rigors of growth in a high-slope environment. Buttressing was rarely seen in *O. usambarensis*, across all altitudinal zones (personal observation, Long). The lack of buttress supports may also be a limiting factor in distribution in respect to slope.

For macro-level distribution, Poisson's test verifies my earlier prediction and shows massive evidence of clumping within the sampled population, regardless of topography, slope, or altitude (Figure 8; Appendix E for p-value). Microsite preference is having an effect on the clumped distribution. *O. usambarensis* shows a preference for both convex areas and areas with intermediate degrees of slope, in terms of abundance (Figure 5 and 7). Altitudinal preference was also been shown (Figure 3). Unfortunately, the size of the sampled population is too small for a Poisson's Test to be run in respect to topography, slope, or individual altitude zones. However, we can infer from the site preference demonstrated by *O. usambarensis* that this spatial clumping is taking place in these preferred areas.

Another explanation is applicable for the clumped spatial distribution. Seed predation, first suggested by Daniel Janzen, holds that seed predators are more likely to frequent areas near parent trees, producing a distance-dependent effect on seed survival. The idea here is that a ring of seeds will be created at some radius from the parent tree that has the best chance of survival (Terborgh 1992). In the case of common species, these seed predation zones fuse with each other due to the close proximity of parent trees, leaving a hyperdispersed distribution scheme. In rarer species, this fusing of predation zones is absent, resulting in a spatially aggregated distribution (Terborgh 1992). Under this view, this is evidence that *O. usambarensis*, if it was not before, is becoming more rare. Neither of these explanations can be completely discounted here; thus, a combination of these two factors-seed predation and microsite preference-is causing the spatial aggregation of the *O. usambarensis* population in the Mazumbai Forest Reserve. Further work needs to be done to disentangle the details of this.

The interactive aspect of the ANOVAs yielded unexpected results. For abundance, slope, topography, and altitude were all shown to be statistically significant on a micro-scale when considered independently. Yet, when these three factors are acting together, they have an insignificant effect on abundance (Table 1). It is logical to assume that the areas which were significantly preferred when considered individually would also be significantly affecting abundance when considered together. If the interactive aspect of the ANOVA is correct, then convex areas with an intermediate degree of slope contained in the 1500-1600 masl zone (the preferred areas when considered individually) are not necessary the physical environments most conducive for the success of *O. usambarensis.*

However, not all results from the interactive ANOVA tests were unexpected. The interactive effect of topography and altitude was found to have a significant impact on abundance (Table 1). DBH was also shown to be significantly affected by topography and altitude acting in concert (Table 1). In the case of abundance, it may be possible that the addition of slope is negating the significance

when considered with the other two independent variables. For DBH, slope was individually insignificant so the statistical insignificance of the combined effects of all of the variables needs no accounting for. It is possible that the statistical instrument, the ANOVA, is not best suited to investigate the interactive effects of three independent variables on a single independent one. As a result, these interactive results must be examined with a skeptical eye, and more faith should be put into the individual results.

It is worth mention that a similar study conducted by Cohen (2006), working with the same population within the same sample frame, yielded almost completely contradictory results with this study. Significance was established with the macro-level variables, but not with the micro-level variables, which is the exact converse of this study's results. Cohen found statistically robust linear relationships were found across altitudinal gradients in respect to both size and density. Although significance was not claimed, 64% and 67%, respectively, of the changes in density and size were found to be contributable to altitude. I attribute these differences to methodological reasons. The non-random site selection of the study could have biased the study favor of environmental conditions where *O. usambarensis* actually occurs. It is unlikely that plots were sampled where *O. usambarensis* was absent due to the nature of non-random plot distribution. Also, a lesser area was sampled (28,800 m^2 versus 540,000 m^2) and the earlier study compiled distribution data on two species, *Syzygium guineense* and *Ocotea usambarensis*, possibly masking the species-specific biogeography of either.

The micro-scale altitude preference of *O. usambarensis* found by this study does correspond to Panteleo's attitudinally determined bands of community composition (2001). Pantaleo found that O. *usambarensis* was dominant at 1430-1880 masl and 1570-1910masl, which agrees with this study's determination of a preferred altitude of 1500-1600masl.

Conclusion

This study aimed to use the biogeography of *O. usambarensis* as an indicator of the incredible amount of macro and micro scales of heterogeneity in terms of physical gradients within high diversity areas, such as montane rainforests, and their subsequent effect on tree distribution and growth. Altitude was not significantly affecting DBH or density across all sampled altitudinal zones, yet despite this lack of a linear correlation, an altitudinal site preference was still shown. This shows that the relationship between *O. usambarensis* and altitude may not be as clear cut as previously thought and is indicative of site selection in respect to geographic gradients.

Topography significantly affected size, and topography and slope significantly affected density with the likely key being local hydrology and the resulting structural concerns for the trees. This shows that small-scale environmental heterogeneity is a key determinant for the size and distribution in *O. usambarensis*. This indicates the capacity of purely geographic factors to maintain non-uniformity in the forest through the creation of species-specific preferred sites. Forest diversity and the survival of the forest as a true mosaic of species can be largely attributed to the effects of spatial heterogeneity acting in multiple scales.

Future research should be undertaken in the Mazumbai Forest Reserve in order to further quantify the effects of small-scale heterogeneity in montane rainforests. It would be interesting to study the hydrology of the Reserve in terms of vegetation cover and community composition. Gap disturbance and its relationship to avian diversity would also be an interesting topic to investigate.

Temporal heterogeneity in respect to arboreal biogeography should be studied in the future to supplement this study and to provide answers to the effects of local-scale geography as it varies through time.

Acknowledgments

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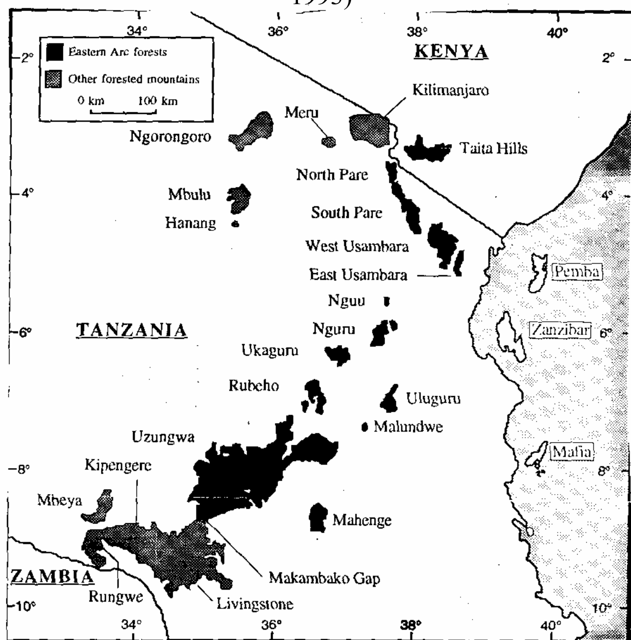
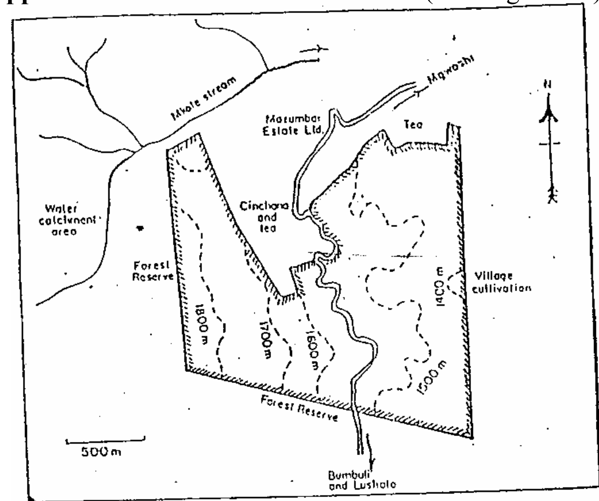
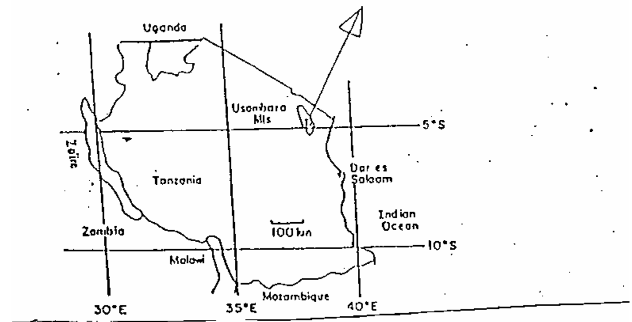
Appendices

Appendix A: The Eastern Arc Mountains (Lovett and Wasser 1993)



Appendix B: Mazumbai Forest Reserve (Terborgh 1992)





Appendix C: Datasheet Scheme

Plot data

Plot #	Quadrat #	Quad. Topo	Quad Slope	Metadata	Altitude

Ocotea data

Plot#	Quadrat #	Immediate Topo	Immediate Slope	DBH

Appendix D: P-values for Three-Way ANOVAs
ANOVA: Dependent Variable, DBH

Slope	.274
Topography	.003
Altitude	<.000
Slope*Topography	.141
Slope*Altitude	.071
Topography*Altitude	.001
Slope*Topography*Altitude	.78

Tukey Post-Hoc, Topography

1 (concave)	2 (intermediate)	<.000
	3 (convex)	.004

ANOVA: Dependent Variable, Number of Individuals

Slope	.043
Topography	<.000
Altitude	<.000
Slope*Topography	.610
Slope*Altitude	.494
Topography*Altitude	.022
Slope*Topography*Altitude	.370

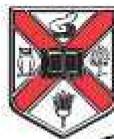
Appendix E: Chi-Square Test of Poisson Distribution

P-value = 4.9E-203

Observed: Number per Quadrat	Frequency	Expected: Number per Quadrat	Frequency
0	112	0	12
1	21	1	35
2	11	2	50
3	11	3	48
4	8	4	35
5	8	5	20
6	6	6	10
7	8	7	4
8	11	8	1
9	2	9	0
10	3	10	0
11	4	11	0
12	1	12	0
13	1	13	0
14	2	14	0
15	1	15	0
16	2	16	0
17	0	17	0
18	0	18	0
19	2	19	0
20	0	20	0
21	0	21	0
22	0	22	0
23	0	23	0
24	0	24	0
25	0	25	0
26	0	26	0
27	0	27	0
28	1	28	0
29	0	29	0
30	0	30	0
31	0	31	0
32	0	32	0
33	0	33	0
34	0	34	0
35	0	35	0
36	0	36	0
37	0	37	0
38	1	38	0

Poisson probability formula for expected values (Zar 1999):

$P(X) = (P(X-1)\mu)/X$
$P(0) = e^{-\mu}$
μ = number of individuals / number of discrete units



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