Applicant:	Dr. Elizabeth Hughes
Primary Supervisor:	Dr. Edward Wallace
Secondary Supervisor:	Dr. Elizabeth Bayne
Host Organization:	Institute for Cell Biology, School of Biological Sciences, University of Edinburgh.
Project Title:	Dynamic mRNA processing in response to environmental stimuli in the fungal pathogen *Cryptococcus neoformans*.

2. Reasons for applying for a fellowship

I am a skilled and highly motivated molecular biologist with 10 years lab-based experience, 6 post-doctoral. In order to succeed in the current job market, I need to update my skillset and access the rapidly advancing technologies and evolving practices in my field.

My PhD investigated the relationship of HIV viral populations and disease progression via evolutionary analysis of isolates infecting lymphoid and non-lymphoid tissues [1,2]. My first author paper reported the discovery of a previously unknown dormant HIV population in the brain and has over 100 citations to date. [1] As a post-doc, I examined Hepatitis C Virus replicative processes by investigating a non-structural protein (NS5B) implicated in RNA-dependent-RNA-polymerase activity. I optimized bacterial expression systems and purified this protein. I also investigated the structure/function of the major- outer-membrane-proteins of *Chlamydia trachomatis* and *psittaci* where I cloned and expressed wild-type and mutated proteins and functionally reconstituted them at the single-channel level.[3] I then joined a leading bio-safety testing facility, BioReliance, as the research and development scientist and developed a wide range of molecular based assays for clients while also providing technical training/support for colleagues.

I took a career break at the end of April 2004 when my daughter was 18 months old to raise my family. My husband works in the marine industry as a consultant which regularly takes him away from home for extended periods of time. Due to this and prohibitive childcare costs we decided I would put my career on hold and take on the administrative side of my husband's business. During this time I have been an active member of primary and secondary school councils and have volunteered as a teacher's assistant at Lanark Primary School for general science and as a group leader for cub pack holidays and weekly meetings for cubs and scouts. I have been an active fundraiser through school parent councils and local youth groups. My children (16 and 13 years) now attend high school and I feel it is the right time for me to return to my chosen career.

After applying for numerous positions I learned of the Daphne Jackson career re-entry Fellowship from my supervisor Dr. Wallace. This is an ideal platform for me to resume my career because the emphasis is on improving employability through updating existing, and gaining new skills. This fellowship will enable me to become competitive in the highly skilled molecular biology field while still caring for my family. I will gain valuable expertise in new routine technologies such as RNA-seq, which have been developed during my career break, and forging relationships with prospective employers and mentors to ensure the success of my career.

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3. How can an environmental fungus, *C. neoformans*, adapt to cause disease in humans?

Cryptococcus neoformans lives in the environment growing on trees, pigeon droppings and in the soil. It infects people when they breathe it into their lungs (see figure 1). Infection is rare in healthy people and most cases occur in people with a weakened immune system (cancer treatment patients or HIV). In the lung it causes a pneumonia-like-illness but it can spread to the brain causing life-threatening meningitis. Approximately 1 million cases occur globally each year resulting in estimated deaths of 600,000.

How does *C. neoformans* adapt to the rapid change in environment from soil/vegetation to a mammalian lung? I will examine what changes occur in this fungus when it reactivates in a host-like environment and identify observable characteristics under different environmental conditions. I will look at the changes in the amount/type of genes that are switched on and off during exposure to these conditions. Measuring the amount of gene activity can tell us a lot about how an organism responds to its environment.

Once *C. neoformans* has entered the lung it will encounter a number of microbes, some of which will be symbiotic and others that may cause disease. I will investigate the relationship between Cryptococcus and bacteria using different fungal strains and fragments of bacterial cell walls. By observing changes in the characteristics of each fungal strain and correlating these features with changes in the expression of genes I will assess the impact bacterial cell wall fragments have on the ability of Cryptococcus to adapt to a new environment.

Identifying unique or critical pathways Cryptococcus uses to cause disease will provide unique targets for drug design. I will document any changes in Cryptococcus during reactivation in different environmental conditions and study the knock on effects by removing identified critical fungal genes or genes in a critical pathways and examine the effects of these mutant stains during reactivation.

Fungal infections are one of the hardest diseases to manage in humans. Most infect people with underlying problems and jeopardize medical advances in cancer care and organ transplant because the immune system is weakened. With 600,000 deaths caused by *C. neoformans* infections world wide a concerted effort is needed to work out how this organism can change to grow in our lungs and spread through our bodies, avoiding all our defenses, to cause disease.

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Abstract

How does Cryptococcus adapt to the lung environment? Transformation from spores/desiccated yeast into actively replicating cells must be associated with dynamic regulation of mRNA and hence gene expression. This proposal will build on preliminary data from Dr. Wallace/Dr. Ballou showing the addition of serum to media causes a radically different response in phenotype/RNA abundance during reactivation and a distinct morphological change from a normal yeast to a titan cell, replicated using purified muramyl dipeptide. It is not known which component(s) in the media gave rise to such differences/changes. The goal of this proposal is to investigate how Cryptococcus adapts to the lung environment by analyzing what serum component(s) trigger an acute stress response, investigating how bacterial cell wall components influence gene expression, and identifying essential genes and biological function. To achieve this goal *in vitro* culture models will be employed to dissect distinct stimuli and analyze differential-gene-expression (RNA-seq)/function (CRISPR) to provide a snapshot of cellular metabolism providing insights for novel therapeutic methods.

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5. Host Organization

The University of Edinburgh is ranked 18th in the world, 4th in the UK and the top university in Scotland. As one of the UK's leading research facilities it provides a state-of-the-art environment for research. This was reaffirmed by the results of the 2014 Research Excellence Framework placing this University as Scotland's top-ranked research institution. The University of Edinburgh is a member of the SWAN charter and won its first Athena Swan Institutional Bronze Award in 2006 and has just renewed its Silver Award in 2018. The University of Edinburgh has also signed the national Concordat to support the career development of researchers. This document lays out 7 principles to increase the sustainability of research careers in the UK and to improve the quality, quantity and impact of research in society.

The Centre for Synthetic and Systems Biology, in the Institute for Cell Biology, is a unique inter-disciplinary environment with a track record for multi-disciplinary research. I will be joining the Wallace lab in this centre. Dr. Wallace is a new principal investigator at the University of Edinburgh, having established his lab in early 2018, consisting of Dr. Rosey Bayne (PDRA/Lab manager), Dr. Laura Tuck (PDRA) and Samuel Haynes (PhD student). The centre includes the Edinburgh Genome Foundry (a world class facility for automated DNA design and assembly) and EdinOmics providing expertise in quantitative biochemistry including up-scaling and automation of RT-qPCR. There are facilities for cell imaging (LEAP and single cell analysis and microscopy groups) and for innovative data analysis and mechanistic modelling.

My primary supervisor, Dr. Wallace, is a quantitative biologist specializing in fungal RNA processing. My secondary supervisor, Dr. E Bayne, specializing in endogenous RNA interference pathways and is using Cryptococcus as a eukaryotic model. Working with my supervisors will allow me to build on my existing skills in the field of molecular biology whilst learning new techniques including medium throughput RT-qPCR, cDNA library preparation and high throughput sequencing (RNA-seq), lab automation, bioinformatics, and working with large data sets. This will provide me with crucial work experience in one of Scotland's world-class universities and equip me with specific skills in high demand in biomedical research and biotechnology industries.

My collaborator, Dr. Ballou, is an expert in *C. neoformans* biology, including relevant culture conditions and models of host-pathogen interaction required for this proposal. I will spend some time shadowing in the Ballou lab to learn these techniques. Dr. Ballou will contribute advice, training and support my professional development.

Dr. R Bayne has 30 years of experience in molecular biology techniques and will take a lead role in my technical/lab based training.

Table 1: Supervisors and Collaborators

	Name	Institute	Position
Primary Supervisor	Dr. Edward Wallace	The University of Edinburgh	Sir Henry Dale Fellow (Wellcome Trust/Royal Society Early Career Research Fellow). Institute for Cell Biology, School of Biological Sciences
Secondary Supervisor	Dr. Elizabeth Bayne	The University of Edinburgh	Reader in Epigenetics, School of Biological Sciences,
Collaborators	Dr. Elizabeth Ballou	University of Birmingham	Sir Henry Dale Fellow and Lecturer. Institute for Microbiology and Infection, School of Biosciences

Word Count: 432/400

6. Retraining Program

6.1: Technical Skills

In order to carry out the proposed aims of my fellowship application I will have to learn a number of new skills while updating existing ones. I will vastly expand my knowledge of new exciting techniques like RNA-seq and CRISPR while updating existing ones such as qPCR. Learning large scale data set production and analysis will be vital for this project due to the large volume of data that will be produced. The ability to handle and analyze large data sets is becoming an imperative skill in modern biomedical science.

Table 2a: New and Refreshed Technical Skills

Training		
New Skills	Method	Trainor/Course
How to culture and extract RNA from Cryptococcus	1-1	Dr. R Bayne/Dr. Ballou
How to identify and phenotype Cryptococcus using various microscopic techniques and stains	Course/1-1	The Wellcome Centre Microscopy Course run by Dr. Kelly at the University of Edinburgh/Dr. Ballou
Lab automation to enable processing of large numbers of RT-qPCR	1-1	1-1 at the Edinburgh Genome Foundry in the University of Edinburgh
RNA-Seq	1-1	Dr. R Bayne and Dr. Wallace
Bio-Informatics to analyze the large data sets produced	Course/1-1	Bioinformatics for Genomics, a 5 day workshop run at the Edinburgh Genomics Centre in the University of Edinburgh (£750)/Dr. Wallace
CRISPR-Cas 9 system	1-1	DR. R Bayne
Refreshed Skills		
Aseptic technique	1-1	Dr. R Bayne
RNA manipulation	1-1	Dr. R Bayne
RT-qPCR	1-1	Dr. R Bayne and Dr. Wallace
Learn how to use updated tools/equipment for these methods	1-1	Dr. R Bayne and Dr. Wallace

This fellowship will increase my employability by bridging my skills gap using a framework for structured training of relevant skills in demand in today's job market. I will train under the guidance of Dr. Wallace, D. E Bayne, Dr. Ballou and Dr. R Bayne who are proficient in all the necessary techniques and are committed to supporting me and my endeavor to return to the workforce. My training will build on my current skills to update my expertise while developing new in-demand skills and forging new relationships in a work environment.

6.2: Professional Skills/Development

During this fellowship I will have access to 3 training courses from the Daphne Jackson Trust covering professional skills, how to publish and how to improve your confidence. This provides an excellent opportunity for me to develop as a researcher and also as a person. Having been away from science for a number of years, building confidence in myself and my ability is very important.

The Institute for Academic Development at the University of Edinburgh is a facility providing many opportunities for education and professional development. The available program provides workshops and courses on academic writing, career management and development, data management, funding opportunities, ethics, teaching and supervising students. During my fellowship I will use these resources to enhance my professional qualifications/development. Within the Institute for Cell Biology there is also a dynamic seminar series covering a wide range of topics from internal and external speakers.

Table 2b: Personal/Professional Development Opportunities

Course	Method	Provider
Professional skills	Workshop	Daphne Jackson Trust
How to publish	Workshop	Daphne Jackson Trust
How to improve your confidence	Workshop	Daphne Jackson Trust
Finding funding for research	Online	IAD*
Get that paper written and published	Workshop	IAD*
Managing your research data	Workshop	IAD*
Writing research proposals for the college of science and engineering	Workshop	IAD*
Attracting your own research funding	Workshop	IAD*
Be better than boring bullet points: giving a great presentation	Workshop	IAD*
Academic CVs	Workshop	IAD*
Effective collaborations	Workshop	IAD*
Practical project management for researchers	Workshop	IAD*
Professional networking, engaging and strategy	Workshop	IAD*
Project management (research)	Workshop	IAD*
Spotlight on…Co-supervision	Workshop	IAD*
Time management	Workshop	IAD*

*The Institute for Academic Development at the University of Edinburgh

During my training I would also like to attend relevant conferences as these provide a national/international platform for sharing information and ideas and keeping up to date with the latest innovations and advancements. This will allow me to present my work and show what skills I have learnt and network with other research groups. Some upcoming conferences within the field of mycology are:

- British Society for Medical Mycology, Annual.
- Human Fungal Pathogens, Biennial.
- 6th International Conference on Mycology and Fungal Infections, Oct 2019, UAE.
- 11th International Conference on Cryptococcus and Cryptococcosis, 2020, Uganda.
- International Society for Human and Animal Mycology, March 2021, India.

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Table 2c: Re-Training Program Summary

Skill	Methods	Refresh skills	New skills	Month	Base*	Objective
Cell Culture Techniques	Culture preparation/storage	✓	✓	1-3	LB	Refresh knowledge and apply to fungal cells to test the effect different environmental stimuli have on the morphology of Cryptococcal cells, correlate with gene expression.
	Sub culturing/cell-maintenance	✓	✓			
	Aseptic technique	✓	✓			
Microscopic Techniques	Visualization/fixing/staining of fungal cells	✓	✓	1-3	LB	Identify morphological changes in cultured yeast cells grown in the presence of different environmental stimuli.
RNA Manipulation	QIAgen plant and fungal RNA extraction kit		✓	3-6	LB	Gain experience in using up-to-date techniques for the isolation of RNA from fungal cells.
	Assess RNA quality/quantity, Agilent bioanalyzer	✓	✓			
RT-qPCR	Primer design/validation	✓		3-8	LB	SYBR-Green fluorescence based qPCR to analyse gene expression in response to environmental stimuli to provide a deeper understanding of the molecular mechanisms underpinning physiological change.
	RT-qPCR	✓	✓			
	cDNA synthesis	✓				
	Data analysis		✓	9-14	CB	
Lab Automation	RT-qPCR using 384/1536-well plates		✓	3-8	LB	Training in automatic plate loading at Edinburgh Genome Foundry
Next Generation Sequencing	RNA-seq		✓	13-19	LB	Illumina sequencing with random primed cDNA synthesis non-strand specific protocol to quantify the dynamic expression levels in yeast cells under variable conditions. This technique enables novel RNA's to be discovered.
	Data analysis		✓	16-21	CB	
Bio-Informatics	R-Programming		✓	1-24	CB	Use R to analyse RT-qPCR and DESeq2 to analyse RNA-seq data. As required additional analytical methods will be employed.
	Handling/analysis of large data sets		✓	Continuous	CB	

CRISPR-Cas 9 system	Functional analysis of genes		✓			Knock out genes of interest for functional characterization.
Personal Development	Attend courses on scientific writing, bioinformatics, writing for grants/proposals, oral presentations, networking and leadership	✓	✓	Continuous	TBD	Gain valuable transferrable skills for further development of my career and to secure further funding or employment following this fellowship.
Attend Seminars/ Conferences	Expand knowledge base		✓	Continuous	TBD	Network with other scientists to share my research/initiate future possible collaborations.
Meetings with Sponsor	Discuss research project and progress		✓	Bi-annual	OB	Discuss progression of project and training enabling me to keep on track. Implement new training strategies where applicable.
Progression Milestones	Reports/publications		✓	Annual	OB	Provide yearly reports on progression of project and publish results in peer reviewed journals.

*LB-Lab based, OB- Office Based, CB- Computer based, TBD-To be decided

7. Dynamic mRNA processing in response to environmental stimuli in the fungal pathogen *Cryptococcus neoformans*.

Background:

C. neoformans is an opportunistic facultative saprophyte and an important global human pathogen[4,5]. *C. neoformans* primarily infects immunocompromised individuals, who will have an abnormal lung microbiome, and is one of only a few fungal species that have been shown to cross the blood-brain barrier leading to cryptococcal meningitis that is fatal if left untreated [4,5]. The onset of the AIDS epidemic in the 1980s was accompanied by a surge in cryptococcosis cases world-wide. Antiretroviral therapy and antifungals have reduced the number of fatal cryptococcal meningitis cases, however, it remains a serious concern for the immunocompromised and is a major problem in resource-limited countries, where HIV prevalence is high, and access to health care and appropriate drug regimens is limited. A global number of almost 1 million cases of cryptococcal meningitis are estimated to occur with more than 600,000 resulting deaths.[4,6] Cryptococcus is prevalent in the environment world-wide and over 70% of cases occur in sub-Saharan Africa. However, an outbreak of *Cryptococcus gatti* in immunocompetent individuals has been reported in North America[7–9] and 2 patients in Glasgow were infected and with Cryptococcus in late 2018 where one patient died as a result.

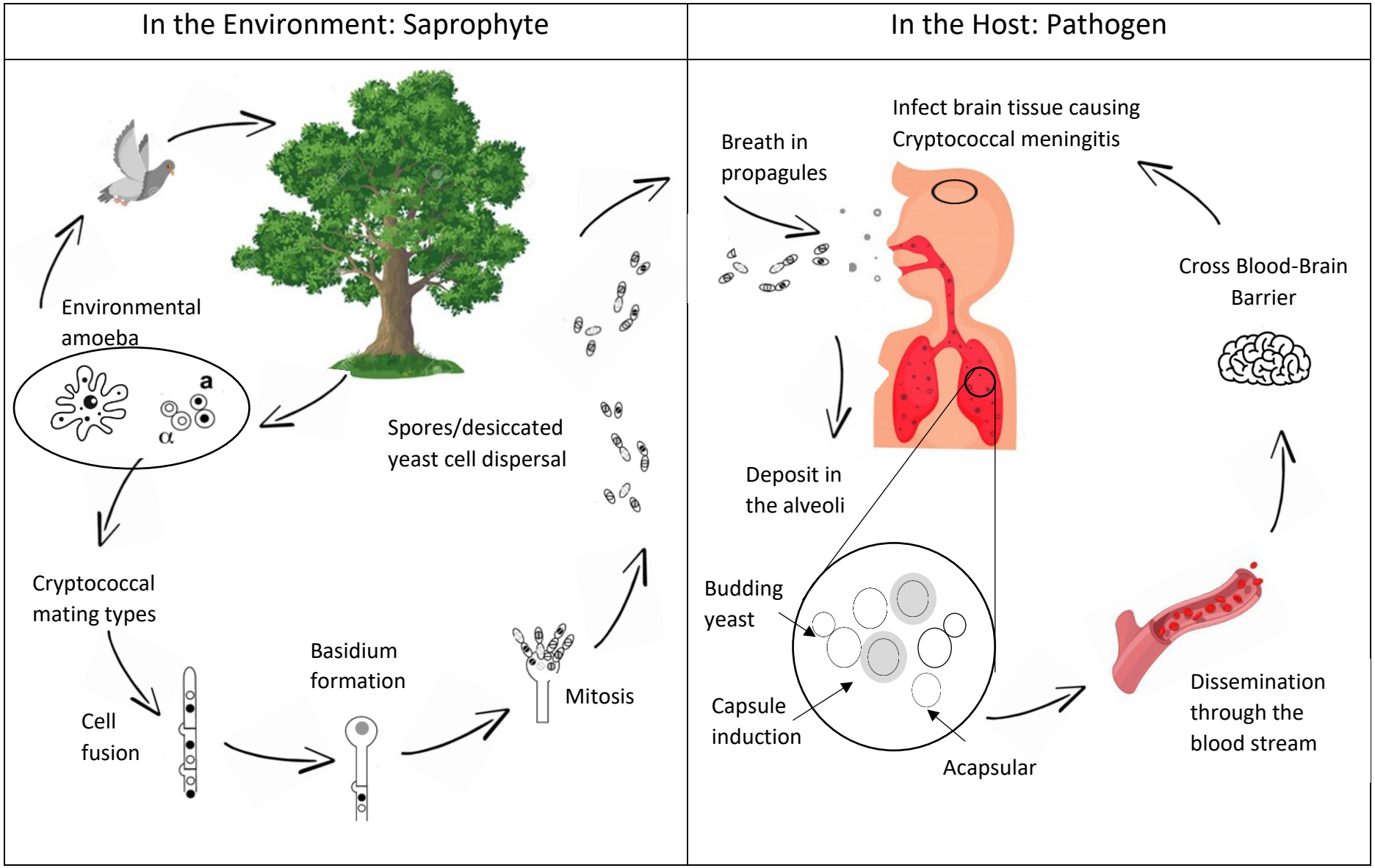
C. neoformans is found in the environment typically associated with pigeon guano, soil and decaying wood. It is a free living fungus with no requirement for a mammalian host, however, it is able to adapt, survive and proliferate within mammals to cause disease. Inhalation of spore/desiccated yeast cells are postulated to act as infectious propagules as only particles smaller than 5µm in diameter can reach the alveoli. The natural ecology and long-term disease progression of *C. neoformans* is beginning to be characterized, however, there is very little understanding of the early stages of *C. neoformans* infection. *C. neoformans* must undergo rapid changes in gene expression to adapt to the alien environment and microbiome of the lung and its ability to alter its transcriptome in response to different environmental stimuli will ensure its survival in different host niches. Indeed not many fungi can grow at the human body temperature of 37^{o}C, a characteristic virulence factor of *C. neoformans* and consistent with its role as a human pathogen [10].

I will examine what happens when this organism reactivates within this alien environment by measuring differential gene expression under different conditions and relating this to the main phenotypic and virulence factor, capsule production. Measuring changes in gene expression provides a snapshot of what is happening inside the cell and by doing this over a time course we can follow the metabolic activity within a cell and essentially see what it is planning. We can then formulate a hypothesis on the adaptive response/responses required for this accidental pathogen to survive.

By combining bioinformatics and experimental investigation essential genes can be identified and putative proteins can be characterized. However, knowledge of the mRNA

expression pattern alone does not necessarily indicate protein function and therefore must be experimentally verified. I will apply the CRISPR-Cas 9 system to functionally characterize any genes of interest. A deep understanding of the transcriptome and gene function in Cryptococcus may elucidate potential targets for drug design. New anti-fungal treatments are badly needed as the current therapies were developed over 50 years ago. This will require a greater understanding of the basic biology and virulence mechanisms of fungal pathogens.

Figure 1: *Cryptococcus neoformans* life cycle: Saprophyte verses Pathogen



<u>Aim 1</u>: What environmental stimuli in the host trigger an acute stress response in *C. neoformans*?

Current knowledge of the early events in *C. neoformans* infections are based on research using animal models or *in vitro* culture methods, primarily in rich fungal support media such as YPD during log phase growth. While these methods have produced useful data they do not accurately reflect the deposition of desiccated yeast into the lung environment, where nutrients are likely to be scarce.

Previous investigations carried out by Dr. Wallace and Dr. Ballou, to dissect the contributions of host factors and temperature in shaping initial growth, found a distinct physiological response (capsule induction) and differential RNA abundance between different growth conditions (see Fig. 2-4). Different capsule phenotypes have been observed in different organs and *C. neoformans* can modify the size and structure of its capsule in response to environmental stimuli suggesting plasticity of the capsule is of biological importance. The reactivation of Cryptococcus is radically different in rich media compared to cell culture media containing serum suggesting host-like media/serum induces capsule production. However, we do not know which component(s) give rise to this phenotypic change or which pathways are responsible. I will address this gap in knowledge by using well defined media and measuring the transcriptome response of Cryptococcus.

Figure 2: RNA time course to measure the contribution of nutrient and temperature to initial growth.

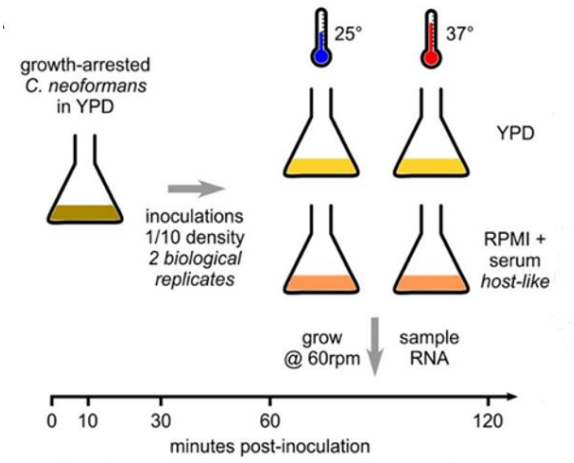


Figure 3: Micrograph with India ink staining 150 min after inoculation. Budding (green arrow) in YPD. Capsule induction (red arrow) in RPMI at 37°C

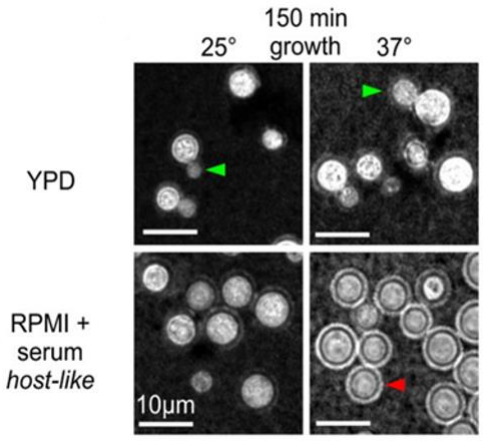


Figure 4: Heat map shows log2 of the difference from median abundance (transcripts/million) for each RNA, log2 mean of 2 biological replicates.

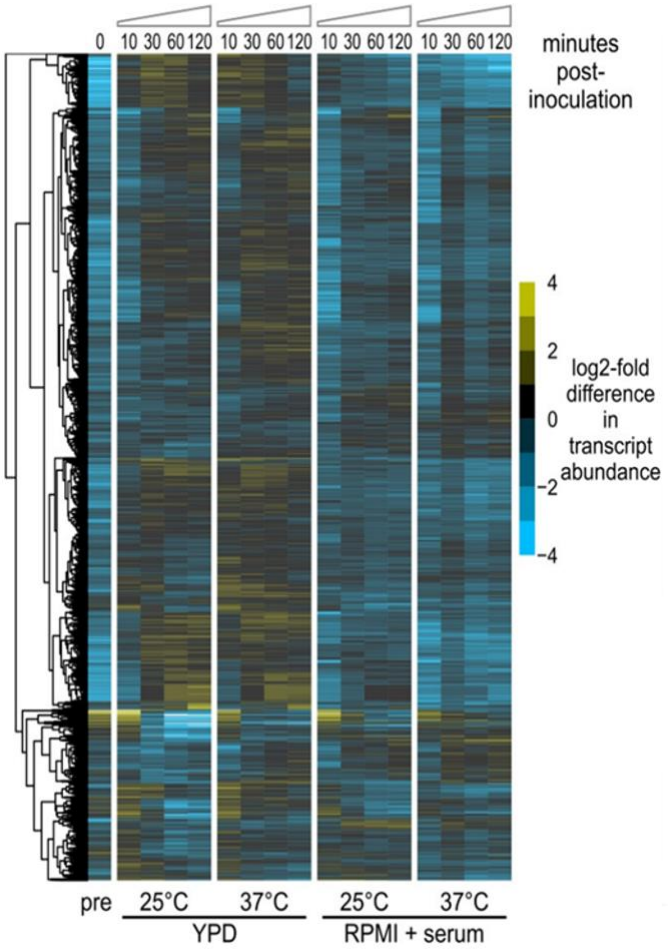
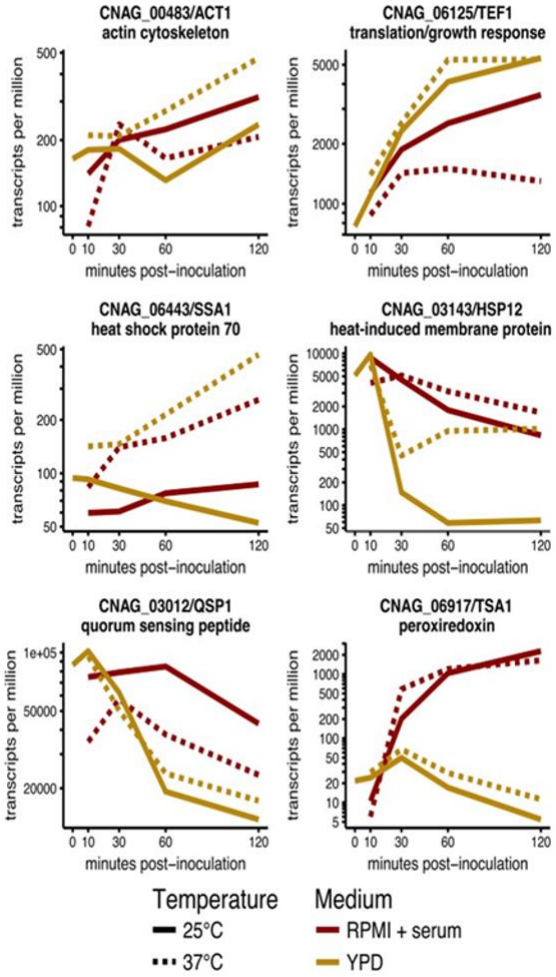


Figure 5: Select genes showing differential regulation of RNA abundance (transcripts/million), log2 mean of 2 biological replicates.



Objective:

In the first 4 months I will retrain in cell culture and microscopy techniques and apply this to growing and identifying yeast cell morphologies. In months 3-12 I will learn how to extract RNA from yeast cells using the QIAgen-plant-and-fungal-RNA-extraction-kit and will identify what causes this phenotypic shift by analyzing differential gene expression using RT-qPCR. I will also be trained in lab automation at the genome foundry to enable me to upscale the RT-qPCR through automatically loaded plates of 1536 wells. This will highlight the important early steps for adaptation to the lung environment and will provide me with an opportunity to further develop my molecular and microbiology skillsets. I will be trained how to use R to analyze large data sets produced in the second half of my first year.

Method:

To determine whether the phenotypic change is due to serum or host-like media in the above experiment I will inoculate growth-arrested *C. neoformans* yeast cells grown in YPD (GA-Cn-YPD) into RPMI-1640 media and YPD + serum and incubate at 25^{o}C and 37^{o}C. I will determine any phenotypic changes (India ink stain for capsule induction) and extract RNA for analysis using RT-qPCR (verified primers against differentially expressed genes previously detected by RNA-seq in the Wallace lab). I will compare alterations in the gene profile in relation to phenotype.

It is known that serum can induce capsule production in Cryptococcus[11] but it is unknown which component(s) of serum cause this response. Although it is routinely used in cell culture serum, a key host-relevant stimulus, it is a variable and undefined component with a complex composition that is still not fully understood. I will dissect the component(s) in serum responsible for capsule induction by incubating GA-Cn-YPD in RPMI-1640 + purified albumin (major component) and RPMI-1640 + charcoal stripped FBS (CS-FBS: reduced levels of endotoxins and hormones; see table 3a below).

Table 3a: Dissection of Serum Components

Media	No Serum (Serum starvation)	FBS	Charcoal stripped FBS	Albumin	25^{o}C	37^{o}C
RPMI-1640	✓				✓	✓
		✓			✓	✓
			✓		TBD	✓
				✓	TBD	✓
YPD	✓				✓	✓
		✓			✓	✓
			TBD		TBD	TBD
				TBD	TBD	TBD

If time allows I will test Minimum Essential Media (MEM) containing electrolyte and carbohydrate levels close to that in human serum and CSF[12]. I will incubate GA-Cn-YPD in MEM with and without serum at 25^{o}C and 37^{o}C and determine any phenotypic changes in capsule induction (India ink stain). Also, RPMI-1640 routinely contains phenol red (pH indicator) which is a weak oestrogen[13] and such steroids have been shown to inhibit *C. neoformans* growth[14] and have shown a synergistic/additive *in vitro* activity with drugs currently used to treat cryptococcosis [15,16] I will investigate the impact that removing phenol red from RPMI-1640 has on capsule induction.

Table 3b: Dissection of Media Components

Media	25°C	37°C
RPMI-1640 + phenol red	✓	✓
RPMI-1640- phenol red	TBD	✓
MEM	✓	✓

<u>Aim 2</u>: **Investigate how bacterial cell wall components influence gene expression in *C. neoformans*.**

Bacteria and fungi coexist and interact in nature competing for space and nutrients. Similar cross-species interactions are expected to occur between the wide range of micro-organisms that constitute the human microbiome[17–19]. Microbial cell wall components are increasingly recognized as important signaling molecules that can inhibit growth and virulence factors and influence morphology of other microbes. The lungs of immunocompromised people are frequently colonized by bacteria and fungi[20] which will disturb the natural microbiome within this organ. The Ballou lab have shown that the serum compound, Muramyl Dipeptide, can modify the morphology of *C. neoformans* from a normal yeast cell to a large polyploid titan cell[21].

How can bacterial cell wall components influence the behavior of co-infecting fungal pathogens? Can these activities be exploited to attenuate fungal virulence and do other bacterial cell wall components have a similar effect? I will investigate the effect of bacterial cell wall components on the induction of capsule in Cryptococcus, one of the main virulence factors and an early morphological response during infection, and relate any phenotypic change to gene regulation. Studying the interactions of bacteria and fungi that colonize the lung in immunocompromised individuals and understanding how these interactions affect pathogenesis will be critical for developing novel methods to prevent these infections.

<u>Objective</u>:

I will examine the transcriptional response of serotypes A and D (H99 and JEC21) to bacterial cell wall components. I will measure changes in gene expression and relate these, using microscopy, to key phenotypes to identify molecular mechanisms underlying these changes. I will extract RNA for analysis using RT-qPCR and RNA-seq (see Fig 6) to identify unknown enriched genes or pathways. This will involve training in RNA-seq (month13-22) and bioinformatics for data analysis using DESeq2 (months 1-36).

Method:

I will identify interactions between the fungal cell surface and bacterial cell wall components by incubating GA-Cn-YPD (fast growing) and GA-Cn-YND (starved) yeast cells in serum-free media (RPMI-1640 and/or MEM depending on results from Aim 1) with different concentrations of purified components of bacterial cell walls:

- Peptidoglycan subunits found in gram positive and gram negative bacteria:
    - N-acetyl glucosamine (NAG)
    - N-acetyl muramic acid (NAM)
    - Muramyl dipeptide (MdP)
- Lipopolysaccharide (LPS) found in gram negative bacteria and
- Teichoic Acid (TA) found in gram positive bacteria.

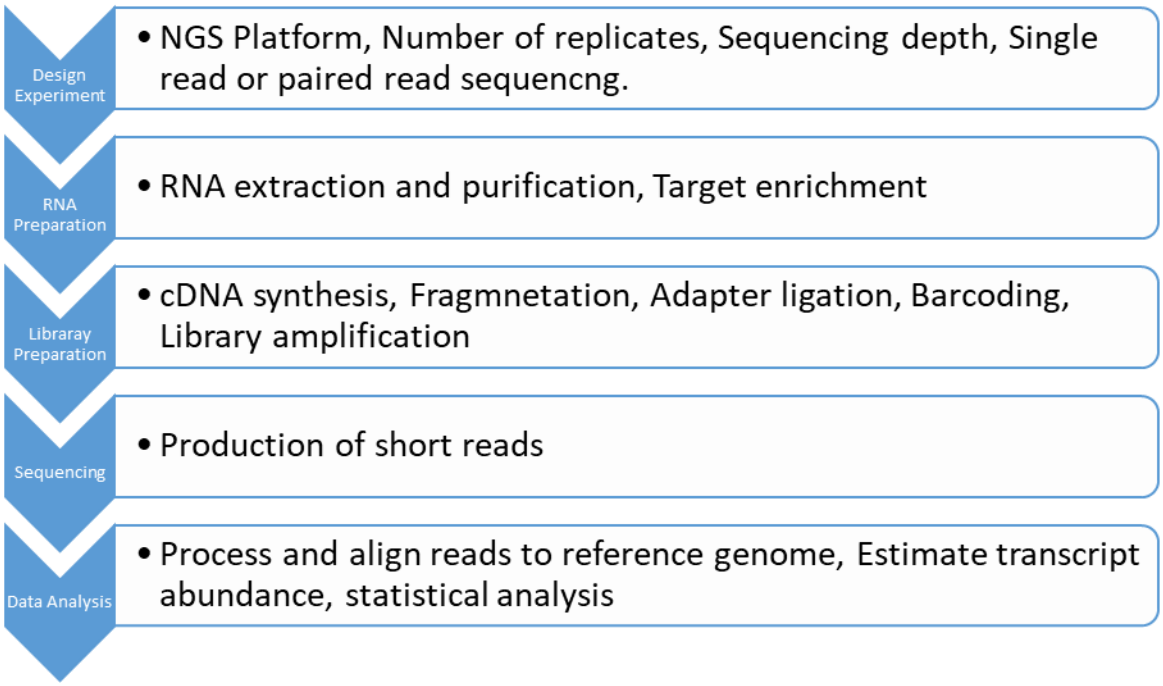
These reagents are all commercially available. I will incubate cells at 25^{o}C and 37^{o}C if capsule was induced at both temperatures in Aim 1. Otherwise, I will only carry out these experiments at 37^{o}C. Depending on results from pilot studies I will further characterize the effect of lipopolysaccharide by incubating yeast cells with the subunits lipid-A (antigenic), core polysaccharide and/or o-polysaccharide.

If time allows I will extend this study to other fungal species of the respiratory microbiome and other surfaces (e.g. *Saccharomyces cerevisiae* and *Candida, Aspergillus, Pneumocystis, Cladosporium, Eurotium, Penicillium sp)* and correlate the results to investigate whether expression of virulence factors is regulated by similar mechanisms/pathways. This will provide preliminary data for further grant applications.

Table 4: Experimental Plan-Aim 2.

RPMI-1640 and/or MEM	Supplemented with 0-20µg of	GA-Cn-YPD (fast growing)		GA-Cn-YND (starved)	
		25°C	37°C	25°C	37°C
	NAG	TBD	✓	TBD	✓
	NAM	TBD	✓	TBD	✓
	MdP	TBD	✓	TBD	✓
	LPS	TBD	✓	TBD	✓
	TA	TBD	✓	TBD	✓

Figure 6: RNA-Seq workflow



<u>Aim 3:</u> Functional characterization of essential genes in *Cryptococcus neoformans.*

<u>Objective:</u>

From the data obtained from Aims 1 and 2 I will use a combination of integrative skills and methodologies to identify essential genes and understand their biological function in *Cryptococcus neoformans* during the early stages of infection. High-throughput sequencing

technology is rapidly becoming the standard method for measuring RNA expression levels and identifying differentially expressed genes in 2 or more conditions. Strategies to characterize gene/protein function fall into 2 multidisciplinary categories:

- Bioinformatics analysis
- Experimental investigation and validation

Bioinformatic techniques assign functional data by searching for well characterized relatives in sequence databases. While this approach has proven to be very successful it must be verified experimentally. The development of advanced genetic tools and the completed sequencing and annotation of Serotype A and D genomes have greatly accelerated the efforts to understand the molecular mechanisms of pathogenesis of this fungus.

Method:

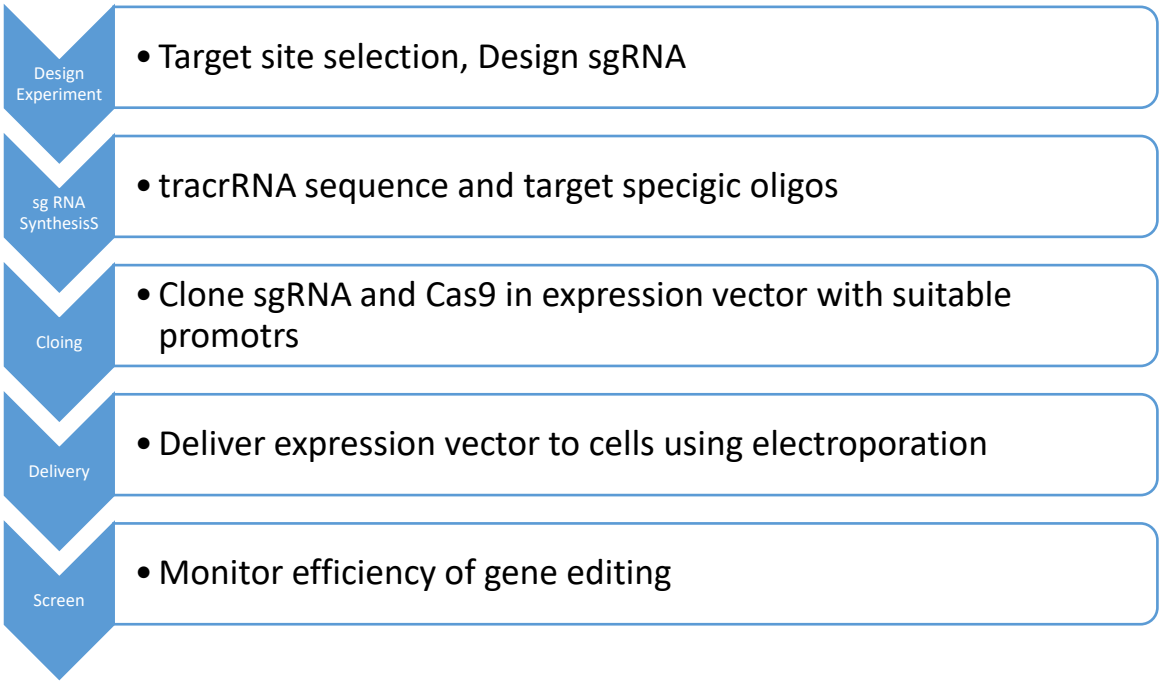
Bioinformatics analysis:

Once high quality data have been obtained, following pre-processing, the reads will be mapped to a reference genome allowing me to infer which transcripts are expressed. This requires no prior knowledge of the transcribed regions or the way in which exons are spliced together and allows the discovery of new transcripts. Quantification will be carried out after normalization of raw read counts by performing statistical analysis to discover changes in expression levels between different environmental conditions using DESeq2. Analysis will be carried out to determine if the differentially expressed genes are associated with a certain biological process or molecular function using Gene Ontology and tools for gene set enrichment and pathway analysis (e.g. DAVID, GSEA, Reactome). These tools compare the frequency of differentially expressed genes with a reference list, usually all the genes in the genome.

Experimental investigation and validation:

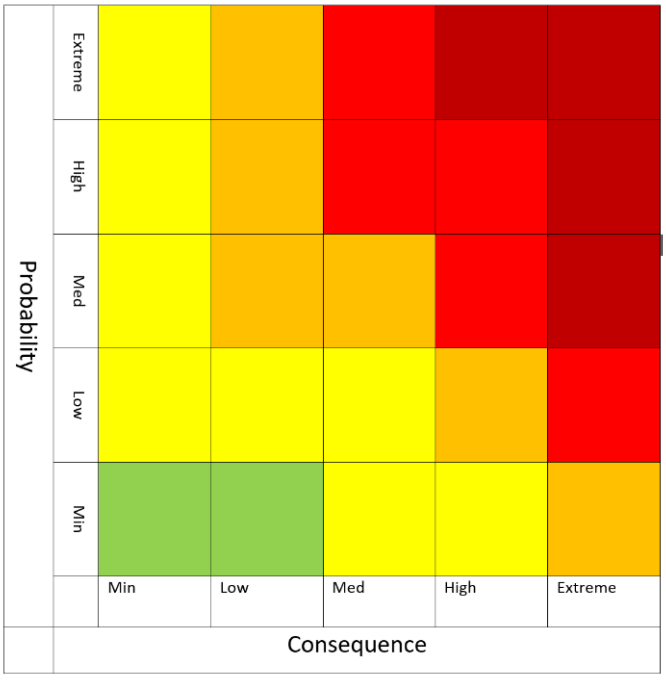
Targeted gene deletions/mutations are necessary to study the function of genes. This enables researchers to switch a gene of interest off, determine the change in phenotype and ultimately deduce the function of the gene product. The CRISPR-Cas 9 system is a powerful tool for genome editing (see Fig 7). I will be trained in the CRISPR based system, established in the Wallace lab, in months 18-24, which will enable me to link genes to functional groups and/or physiological processes. Two studies have shown that CRISPR-Cas 9 technology allows efficient and precise genome editing of Cryptococcus neoformans[22,23]. This technology will be used to knock out genes of interest, highlighted by the bioinformatics analysis, for functional characterization of metabolic and/or signaling interactions between Cryptococcus and environmental stimuli and bacterial cell wall fragments present in the microbiome. Determining the function of genes and exposing critical signaling and/or metabolic pathways may help to identify novel routes for drug design and intervention.

Fig 7: CRISPR-Cas 9 workflow



Risk Assessment:

Table 6: Risk Assessment of procedures



Risk	Risk Level
Extreme	R5
High	R4
Medium	R3
Low	R2
Minimum	R1

Table 6b: Mitigation summary

Process	Hazard	R-M*	Mitigation	R+M**
Handling of *C. neoformans*	Laboratory associated infection	R1	• Category 2 pathogen • Biosafety level 1 • Standard microbiological practices • The Wallace lab has risk assessment in place, including biosafety and genetic modification	R1
Cell culture	Contamination during inoculation, incubation and harvesting	R2	• Use aseptic technique • Use sterile equipment • Use sterile flask stoppers • Use sterile reagents	R1
Lyophilisation of cells	Contamination	R4	• Carried out within a vacuum at -80^{o}C • Use sterile equipment	R1
Reagents used for RNA extraction	Contamination	R2	• All reagents are part of a kit validated by the manufacturer • Use RNA handling techniques such as gloves and RNase free diluents and Eppendorf's	R1
Normalisation of RNA extraction	Inter- and intra-sample variation	R5	• Spike in a 1:100 ratio of methanol-fixed *Schizosaccaromyces pombe,* using three reference genes.	R1
Quality and integrity of extracted RNA	Contamination and/or degradation	R5	• ʎ 260/280 = 1.8-2 (contaminating protein) • ʎ 260/230 = 1.8-2 (contaminating salts) • Assess ribosomal RNA (rRNA) using gel electrophoresis	R1
Removal of genomic DNA from RNA samples	Contaminating genomic DNA	R3	• DNase treat samples • Check quality using nanodrop ʎ260/280 ratio and Agilent bioanalyzer	R1
cDNA Synthesis for RT-qPCR	Contamination with PCR artefacts	R3	• Run 3 biological replicates • Determine whether the same sequences are observed in each • Use appropriate controls; no template and no RT enzyme	R1
Primer design for RT-qPCR	Ensure specificity	R3	• All the primers to be used have been developed and validated in the Wallace labs to show specificity	R1
Reagents for RT-qPCR	Contamination	R3	• All reagents are part of a kit validated by the manufacturer • Use RNA handling techniques such as gloves and RNase free diluents	R1

			• Use RNase free Eppendorf's • Use appropriate controls; no template	
Normalisation of RT-qPCR for data analysis	Misrepresentation of expression profiles obtained	R3	• Select 3-4 stably expressed reference controls • Use the mean to normalise differences in the amount and quality of starting material • Use the mean to normalise the efficiency of the reaction • RT-qPCR will be carried out under MIQE guidelines	R1
Enrich mRNA for RNA-seq	Remove rRNA (80% of RNA will be ribosomal)	R3	• Select for mRNA with oligo-dT- hybridisation • Specific removal of rRNA	R1
Library construction for RNA-seq	Contamination with PCR artefacts	R3	• Run 3 biological replicates • Determine whether the same sequences are observed in each • Use appropriate controls; no template and no RT enzyme	R1
Gene editing with CRISPR	Specificity and efficiency	R4	• Use short guide RNA (sgRNA) design software • Using RNA-seq data carefully select target sites • Carefully consider the sgRNA:Cas 9 concentration • Optimize the delivery method (electroporation)	R1
Training	Ineffective training	R4	• See re-training program • Discuss progression of training with sponsor • Implement new training strategies where applicable	R1
Equipment	Failures	R5	• All equipment is maintained to a high standard within the Wallace labs by the lab manager	R1
Timelines	Failure to complete the project	R5	• Implement good time management and organisational strategy (see Timetable) • Review progress every 6 months with supervisor • Compile yearly reports	R1

*R-M: Risk without mitigation **R+M: Risk with mitigation

The key elements of mitigation are: Implementing good time management
Organisational strategies
6 monthly and yearly reviews/reports
Adherence to the proposed training schedule
Good laboratory practice

These will ensure that this project is carried out to a high standard and will be completed within the timeframe given.

Impact:

This research is discovery driven and will advance knowledge regarding the initial stages of Cryptococcal infection in the lung. Functional profiling under differing stimuli may identify enriched pathways and/or micro-evolutionary adaptations during the early phase of *C. neoformans* infection and pave the way for the development of novel methods to abrogate the infection and dissemination of this fungus. I expect this fellowship to lead to 2 peer reviewed papers, 1 as first author. This research will be presented at conferences and seminars in order to engage the wider community and forge future collaborations.

Further work:

Using preliminary data produced during my fellowship I would like to investigate the possible relationship between fungi and bacteria in the microbiome to see if production of virulence factors in fungi are regulated or in some way modified by interactions with bacteria. Is there a universal or shared regulatory/signaling pathway that can induce the production of virulence factors in fungi? Investigation of how interactions of organisms in the microbiome contribute to disease; through bi-microbial cultures, investigation of cell wall components or the effects small signal peptides produced by microbes, could provide important insights into novel pathways for the development of anti-fungal drugs. It is my intention to build on the experience I gain during this fellowship to develop a research strategy and collaborations that will follow on from this opportunity.

Word count: 2397/2250

8. Ethical approval and licenses

This work is covered by the School of Biological Sciences' ethics assessment ewallac2-0002, "Dynamic RNA processing in adapting fungi". The proposed work will be entirely with model fungi grown in liquid or solid media in laboratory conditions. No work will be conducted with patients, nor with animals, nor with animal/human tissue. There will be no contact with clinical data. *Cryptococcus neoformans* is a category 2 pathogen and the Wallace lab has all risk assessments in place, including biosafety and genetic modification, for the proposed work.

9. Timetable Table 7: Proposed Research and Re-training

Aim	Task	Month																	
		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
Aim 1	Experimental Design																		
	Culture fungi/Microscopy																		
	RNA extraction from fungi																		
	Culture cells/morphology																		
	RNA extraction																		
	RT-qPCR/Automatic loading																		
	R programming																		
	Data analysis for RT-qPCR																		
Aim 2	Experimental Design																		
	Culture cells/morphology																		
	RNA extraction																		
	RT-qPCR/Data Analysis																		
	RNA-Seq/Data Analysis																		
Aim 3	Experimental Design																		
	CRISPR gene editing																		
Outputs	Courses and conferences*																		
	Progress meetings/reports																		
	Publication of work																		
	Future Planning																		

	Training/Re-training
	Research
	Progress Meetings
	Annual Reports

*Daphne Jackson Trust and IAD courses (to be arranged) and appropriate seminars and conferences to present my work

10. References

1. Hughes, E. S., Bell, J. E. & Simmonds, P. Investigation of the dynamics of the spread of human immunodeficiency virus to brain and other tissues by evolutionary analysis of sequences from the p17gag and env genes. *J. Virol.* **71**, 1272–80 (1997).
2. Hughes, E. S., Bell, J. E. & Simmonds, P. Investigation of population diversity of human immunodeficiency virus type 1 in vivo by nucleotide sequencing and length polymorphism analysis of the V1/V2 hypervariable region of env. *J. Gen. Virol.* **78**, 2871–2882 (1997).
3. Hughes, E. S., Shaw, K. M. & Ashley, R. H. Mutagenesis and functional reconstitution of chlamydial major outer membrane proteins: VS4 domains are not required for pore formation but modify channel function. *Infect. Immun.* **69**, 1671–1678 (2001).
4. Park, B. J. *et al.* Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23**, 525–530 (2009).
5. Harrison, T. S. The burden of HIV-associated cryptococcal disease. *AIDS* **23**, 531–532 (2009).
6. Rajasingham, R. *et al.* Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. *Lancet. Infect. Dis.* **17**, 873–881 (2017).
7. May, R. C., Stone, N. R. H., Wiesner, D. L., Bicanic, T. & Nielsen, K. Cryptococcus: from environmental saprophyte to global pathogen. *Nat. Rev. Microbiol.* **14**, 106–17 (2016).
8. Ma, H. *et al.* The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 12980–5 (2009).
9. Byrnes, E. J., Marr, K. A. & Marr, K. A. The Outbreak of Cryptococcus gattii in Western North America: Epidemiology and Clinical Issues. *Curr. Infect. Dis. Rep.* **13**, 256–61 (2011).
10. Perfect, J. R. *Cryptococcus neoformans* : the yeast that likes it hot. *FEMS Yeast Res.* **6**, 463–468 (2006).
11. Zaragoza, O., Fries, B. C. & Casadevall, A. Induction of capsule growth in Cryptococcus neoformans by mammalian serum and CO(2). *Infect. Immun.* **71**, 6155–64 (2003).
12. McKee, T. J. & Komarova, S. V. Is it time to reinvent basic cell culture medium? *Am. J. Physiol. - Cell Physiol.* **312**, C624–C626 (2017).
13. Berthois, Y., Katzenellenbogen, J. A. & Katzenellenbogen, B. S. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 2496–500 (1986).
14. Welshons, W. V., Wolf, M. F., Murphy, C. S. & Jordan, V. C. Estrogenic activity of phenol red. *Mol. Cell. Endocrinol.* **57**, 169–178 (1988).
15. Butts, A. *et al.* Estrogen Receptor Antagonists Are Anti-Cryptococcal Agents That Directly Bind EF Hand Proteins and Synergize with Fluconazole In Vivo. *MBio* **5**, e00765-13 (2014).
16. Mohr, J. A., Long, H., McKown, B. A. & Muchmore, H. G. *In vitro* susceptibility of *Cryptococcus neoformans* to steroids. *Med. Mycol.* **10**, 171–172 (1972).
17. Bauernfeind, A. *et al.* Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. *Infection* **15**, 270–277 (1987).
18. Nikawa, H. *et al.* Alteration of the coadherence of Candida albicans with oral bacteria by dietary sugars. *Oral Microbiol. Immunol.* **16**, 279–283 (2001).
19. Mayer, F. L. & Kronstad, J. W. Disarming Fungal Pathogens: Bacillus safensis Inhibits Virulence Factor Production and Biofilm Formation by Cryptococcus neoformans and Candida albicans. *MBio* **8**, e01537-17 (2017).
20. Peleg, A. Y., Hogan, D. A. & Mylonakis, E. Medically important bacterial–fungal interactions. *Nat. Rev. Microbiol.* **8**, 340–349 (2010).
21. Dambuza, I. M. *et al.* The Cryptococcus neoformans Titan cell is an inducible and regulated morphotype underlying pathogenesis. *PLOS Pathog.* **14**, e1006978 (2018).
22. Wang, Y. *et al.* A 'suicide' CRISPR-Cas9 system to promote gene deletion and restoration by electroporation in Cryptococcus neoformans. *Sci. Rep.* **6**, 31145 (2016).
23. Arras, S. D. M. *et al.* Targeted Genome Editing via CRISPR in the Pathogen Cryptococcus neoformans. *PLoS One* **11**, e0164322 (2016).

11. Future Planning

During this fellowship I will update technical skills such as cell culture, RNA extraction and manipulation and RT-qPCR. I will also develop new skills to enable me to compete in today's research marketplace. These include next generation sequencing, lab automation and bioinformatics; all of which will be invaluable following on from this fellowship. Having the opportunity to develop professional and personal skills throughout this fellowship is vital to build my confidence as a researcher and network with possible future employers and/or collaborators. I will build my CV and boost my research and publication portfolio while working alongside world class researchers at the University of Edinburgh. This will give me valuable exposure to the unique work environment and culture in research as well as the technical and soft skills needed to succeed in a research driven career.

By the end of the fellowship I would like to be in a position where I can be competitive by securing a good publication record and build up my scientific profile by participating in conferences, attending seminars and continuing to educate myself. My children will be 16 and 19 years and as such I will be looking for full time positions at this stage.

Plan A:

Following this fellowship I would like to continue in research. My first choice would be to write a project grant as researcher co-investigator (BBSRC, MRC, Springboard Award, Wellcome trust career re-entry) with Dr. Wallace to continue my research in *C. neoformans* at the University of Edinburgh.

Plan B:

If I am unsuccessful securing funding to continue my research at the University of Edinburgh I will look for full time PDRA positions in an academic/research setting in central Scotland as my son will still be attending school in Lanark at this time (see Table 7 below). Within the University of Edinburgh there are three labs, including the Wallace labs, working on Cryptococcus; The Bayne Lab, Dr. E Bayne is my second supervisor, and The Hardwick lab. On completion of my fellowship it is possible a post- doctoral position may be available in one of these labs.

Table 8a: Plan B

Institute	Group	Research Interests
University of Edinburgh	Bayne Lab	RNA directed chromatin modification and endogenous RNAi pathways in Cryptococcus.
	Hardwick Lab	Cryptococcus cell division and aneuploidy in host relevant stress conditions
	Amy Buck	Small RNAs in host-pathogen systems
	Alex Rowe	Virulence factors of the malaria parasite and susceptibility in the host
Roslin Institute	Digard Lab	Viral factors controlling the replication/pathogenesis and spread of Influenza A virus
	Prof B. Dutia	Molecular pathogenies of herpes virus and Influenza virus infections
	Abigail Dick	Human and animal prion disease pathogenesis and strain characterization
	Neil Mabbott	Molecular mechanisms underlying the pathogenesis of infectious and neurological diseases of ruminant livestock
Glasgow University	Mclauchlan Group	The functions of virus and host components during Hepatitis C infection
	Thomson Group	Investigating the mechanism behind spontaneous clearance in order to advance the search for a vaccine using next generation sequencing
	Hutchinson Group	Molecular biology of Influenza viruses and the interactions of viral and host proteins during infection
	Viral genomics and bioinformatics group	Analysis of high throughput sequencing data to address viral genomics, transcriptomics, diversity, evolution and transmission
	Dr. R Davies	Molecular interactions between bacterial pathogens and their host outer membrane proteins

Plan C:

My long term goal is to remain in research, however, if this is not feasible I will look to industry/biotechnology companies within central Scotland. (See Table 8 below).

Table 8b: Plan C

Area/Science Park	Company	Interest
Glasgow	Merck Life Sciences	Biosafety testing
Edinburgh	Mordun Research Institute	Proteomics facility
Roslin	Ingenza	Biotechnology
Penicuik	Ab Biotechnology Ltd Services	Product/process development and manufacturing
Elvingston science centre	Charles River Laboratories Ltd	Multi- National Pharmaceuticals Company
Pentlands Science Park	BioReliance Ltd	R&D and testing of high-technology pharmaceutical products
	Moredun Research Institute	Government-supported animal disease research establishment
	Moredun Scientific Ltd	Contract R&D company
Roslin Biocentre	Censo Biotechnologies Ltd	Stem cell technology, contract research services for drug discovery, toxicity testing and cell banking
Stirling University Innovation Park	BioReliance Ltd	Biosafety testing
	Symbiosis Pharmaceutical Services	Drug product testing
	Medpace	Biosafety testing

With the experience I will gain through this fellowship I believe I will become a highly desirable candidate for future employment due to the high quality and world class resources available to me at the University of Edinburgh.

Word count: 413/400