MECHANISMS OF MILTEFOSINE RESISTANCE IN *LEISHMANIA MAJOR*

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Abstract

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In this dissertation, I address development of resistance to treatment with miltefosine in *Leishmania major*. *L. major* is the causative agent of cutaneous leishmaniasis, a disease that faces many difficulties in surveillance and control. Current treatments are expensive and difficult to administer, requiring daily administration from a health care professional for a one-month period. Additionally, none of the currently available therapeutic options for treatment of leishmaniasis were developed specifically for the disease, leading to many severe side effects. Control is further complicated by the burden of developing resistance to pentavalent antimonials, the most common treatment in endemic areas. The most recent treatment option, miltefosine, follows an oral administration that increases appeal of this particular antileishmanial agent. However, upon further investigation, resistance to this particular option has been observed in clinical trials, and resistant strains are easily created in a laboratory environment. Miltefosine-resistant cultures of *L. major* were created through step-wise selection. These drug-resistant populations were then characterized as compared to the
wild-type populations to determine any differences in parasite infectivity, virulence, and overall fitness. Miltefosine-resistant populations demonstrated higher rates of metacyclogenesis, but attenuated virulence in an *in vivo* environment. Once the parasite life cycle had been fully characterized, we further investigated mechanisms of action of miltefosine specifically in *Leishmania*. In cancer, miltefosine is known to induce an apoptotic cell death. In *Leishmania*, the drug appears to have a similar effect, inducing oxidative stress to activate an apoptotic-like mechanism resulting in cell death.
You are a child of the universe, no less than the trees and the stars;
you have a right to be here.
And whether or not it is clear to you, no doubt the universe is unfolding as it should.
- Max Ehrmann, *Desiderata*, 1927

This is for my family and friends; may you find joy in the littlest things in life, especially a well-placed pun.
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1.1 Leishmaniasis Background and Disease Epidemiology

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*, and presents as a variety of clinical manifestations ranging from lesions on the skin to disseminated visceral infections. With an annual global incidence of 2 million cases and a prevalence of more than 12 million, leishmaniasis is responsible for at least 50-70,000 deaths annually. More than 88 countries have reported infection, resulting in 350 million individuals at risk for infection and an estimated 2.4 million disability-adjusted life years (DALYs, years of life lost due to disability or early death). These statistics are grossly underestimated due to misdiagnosis and insufficient disease surveillance systems.

Clinical outcome severity depends on the species of *Leishmania* responsible for infection as well as the immune state of the mammalian host. Visceral Leishmaniasis, a systemic infection (Figure 1.1, top left), is fatal when left untreated. Characterized by weight loss, splenomegaly, hepatomegaly, anaemia, and fever, VL is highly endemic in India and East Africa, with additional cases reported in Bangladesh and Brazil (Figure 1.2, top).
Figure 1.1: Clinical manifestations of leishmaniasis. Visceral leishmaniasis (VL, top left), mucocutaneous leishmaniasis (MCL, top right), and cutaneous leishmaniasis (CL, bottom).
Figure 1.2: Global distribution and status of endemicity of the leishmaniases.
Mucocutaneous Leishmaniasis (MCL) distribution is not as widespread as VL, with nearly 90% of cases reported in South America (Bolivia, Brazil, and Peru, specifically).\(^6\) MCL presents as permanent facial disfiguration resulting from infection of the mucous membranes (Figure 1.1, top right), and is caused by species such as \textit{L. braziliensis}.\(^7\)

Finally, Cutaneous Leishmaniasis (CL) is the most common form, accounting for approximately 1 million new cases each year. CL often characterized by ulcerative lesions that can resolve on their own in the absence of treatment (Figure 1.1, bottom). Many of the symptoms of CL mimic other ailments (such as the common cold and insect bites, to name a few), which contribute largely to misdiagnosis and an underrepresentation of the disease presence. The focus of this dissertation is \textit{L. major}, one of the causative agents of CL.\(^7\) While 95% of CL cases occur in the Americas, 67% of cases are reported in Afghanistan, Algeria, Brazil, Colombia, Iran, and the Syrian Republic, giving CL a global presence (Figure 1.2, bottom).

1.2 \textit{Leishmania} Life Cycle

\textit{Leishmania} species have a digenetic life cycle including both extracellular promastigote and obligate intracellular amastigotes forms (Figure 1.3).\(^3,4\) The 21 different \textit{Leishmania} species are transmitted by approximately 30 different species of phlebotomine sandflies.\(^7\) Similar to other insect vector-borne diseases, only the female sandfly is responsible for disease propagation and transmission. During the proliferating stages of the life cycle, extracellular flagellated promastigotes reside in the gut of the phlebotomine sandfly vector.\(^7,8\) Maturation of these non-infective (procyclic)
promastigotes take approximately 10 days to complete, and the infective (metacyclic) promastigotes will migrate to the proboscis of the sandfly and transmitted to a mammalian host during a blood meal. Following infection in humans, promastigotes are engulfed by macrophages where they differentiate into non-motile amastigotes in the lysosome.⁹ Infected macrophages eventually burst releasing amastigotes to be phagocytized by other macrophages, propagating infection.⁸

1.3 Disease Prevention and Control

Currently, there is no human-safe vaccine.¹⁻⁴ Many attempts have been made to create a successful vaccine for the prevention of leishmaniasis. Whole killed or attenuated parasites and parasite extracts have all been used as potential vaccine candidates, though as of yet there is no apparent success.⁷,¹⁰ Alternatively, on the therapeutic front, vaccines using killed *L. amazonensis* coupled with low-dose antileishmanials (antimonials) have demonstrated promise as a potential chemotherapeutic.¹⁰ Similar success was seen in Sudan when using an injection cocktail of killed *L. major* adsorbed on alum with BCG, resulting in cure rate of 87% (from 53%).¹⁰

Alternatively, some protective effects have been observed through the utilization of sandfly saliva as a form of immunity against leishmaniasis infection. When challenged with a controlled *L. major* infection, non-human-primates immunized with a sandfly salivary protein presented with decreased lesion size, parasites/lesion, and overall disease burden.¹¹ Utilization of sandfly salivary proteins as a form of vaccination against leishmaniasis is complicated by regional antigenic variation of sandfly
Figure 1.3: Leishmaniasis life cycle.
populations. Moreover, the shielding effects from sandfly saliva appear to be limited to short-term protection, and appear to be restricted to immediate infection post-exposure.

Management of leishmaniasis is further complicated by the housing and sanitation conditions associated with poverty, as they create an ideal environment for the sandfly vector. Many of the regions where leishmaniasis is endemic are comprised of people surviving on less than $1 USD per day. Treatment options aside, low-income areas are often subject to increased disease progression along with higher morbidity and mortality. High drug costs (can be up to $1500 USD) coupled with difficulty of treatment administration and emerging drug resistance further complicate these measures to control this disease.

1.4 Current Antileishmanial Agents

Current treatments are expensive, have toxic side effects, and until recently, drug administration has been limited to repeated on-site injections by trained health care professionals. Additionally, the mechanism of action (MOA) is not well understood for leishmania treatments. One of the first-line treatments of leishmaniasis is pentavalent antimonials. The side effects can include malaise, anorexia, and vomiting, and the treatment is administered by daily injections with the drug. Discovered in the 1940s, antimonial treatments have been met with increasing levels of resistance leading to a failure rate of approximately 65%. In the event of antimonial-resistance, the preferred second-line treatment is amphotericin B, an
expensive and equally toxic option.\textsuperscript{1,4,17,18} Paromomycin and pentamidine are additional second-line treatment options for leishmaniasis. Paromomycin was reintroduced in the 1980s\textsuperscript{15} and has demonstrated comparable success to amphotericin B in the treatment of leishmaniasis.\textsuperscript{4,17} Pentamidine has been used as an antileishmanial agent for VL and CL for over 40 years,\textsuperscript{5} though its toxicity and limited availability result in its infrequent use as a treatment option.\textsuperscript{4}

Pentavalent antimonials, commonly meglumine antimoniate/Glucantime or sodium stibogluconate/Pentostam,\textsuperscript{2,3} have been in circulation as the preferred primary treatment for leishmaniasis for over six decades.\textsuperscript{3-5} Treatment is generally administered parentally (daily injections from a medical professional) for 20-30 days and is accompanied by typical side-effects ranging from nausea, vomiting, and diarrhea to hepatotoxicity and cardiotoxicity.\textsuperscript{2,3} For many parts of the world, over 95% of previously untreated patients diagnosed with leishmaniasis will respond to this line of treatment.\textsuperscript{5} However, over the past 15 years, resistance has started emerge – specifically in North Bihar, India. Following several modifications to the existing antimonial treatment regimen (alterations to dosage, frequency of administration, and duration of treatment) it was found that only 64% of patients could be cured in this hyper-endemic region.\textsuperscript{5} This resistance has started to spread to areas of Nepal.\textsuperscript{5} Development of resistance can largely be attributed to drug misuse resulting from lower levels of poverty and illiteracy in endemic areas.\textsuperscript{5} Despite its long-term use, the mechanism of action (MOA) of pentavalent antimonials in \textit{Leishmania} is only generally understood. Studies have
suggested that the MOA involves the inhibition of glycolysis and fatty acid β-oxidation, resulting in alterations to energy metabolism.\textsuperscript{4,5}

Like pentavalent antimonials, amphotericin B (AmB) has been used as a second-line treatment since the 1960s.\textsuperscript{5} AmB also follows a parental administration for approximately 30 days, and common side effects include infusion reactions (high fever, rigor, and chills), renal insufficiency and electrolyte abnormalities.\textsuperscript{3,10} Treatment with AmB is generally limited due to high costs of the drug, ranging from $162-229 USD per patient.\textsuperscript{3,5,10} Selectivity of AmB in trypanosomes (\textit{Leishmania} and \textit{Trypanosoma cruzi}) is due to affinity of the drug for ergosterol, as opposed to cholesterol in mammalian hosts.\textsuperscript{5} Resistance to treatment with AmB is rarely observed, and has been reported as species dependent.\textsuperscript{5} Though resistance to this treatment is rare, increased usage of AmB (as a result of antimony resistance) could lead to the emergence of AmB resistance.\textsuperscript{5}

Paromomycin is more commonly used in the treatment of CL, as it is currently the cheapest treatment option at approximately $7.4 USD per patient.\textsuperscript{10} Further, no threat of clinical resistance has been reported as of yet.\textsuperscript{5} However, early trials with this treatment in relapse patients show significant decreases in sensitivity to this drug.\textsuperscript{5} Side effects for this treatment include ototoxicity, vestibular instability, and nephrotoxicity.\textsuperscript{3} The MOA of paromomycin has been well outlined in bacteria, and points to inhibition of protein synthesis.\textsuperscript{5} No such description exists for the interactions observed in \textit{Leishmania}, and in early studies of resistance to this drug parasites exhibited decreased drug uptake, and potentially inhibition protein synthesis.\textsuperscript{4,5} If paromomycin usage
increases, investigations are warranted to detail a specific mechanism of resistance (MOR) to this treatment.\textsuperscript{5}

Pentamidine is not a commonly used treatment option, but has been available for over 40 years.\textsuperscript{5} After a decade of use in India, response rates to treatment with pentamidine dropped from >95\% to <70\%, suggesting that resistance could easily develop with prolonged use.\textsuperscript{5} There are many severe side effects to treatment with pentamidine, including (but not limited to) hypoglycemia, liver enzyme abnormalities, bone marrow effects (leukopenia, anemia), nephrotoxicity, and cardiotoxicity (arrhythmias, heart failure).\textsuperscript{3} The MOA is very poorly understood for this particular treatment, but the drug affects the biosynthesis of macromolecules including DNA, RNA, phospholipids, and proteins.\textsuperscript{3,5} Early analyses of pentamidine resistance have found decreased uptake and increased efflux, suggesting alterations to membrane transport systems.\textsuperscript{4} Other data, however, have given importance to the role of mitochondria in the mechanism of resistance for this drug.\textsuperscript{5}

1.5 Emerging Antileishmanial Agents

Miltefosine (hexadecylphosphocholine, MIL), registered as a treatment for leishmaniasis in 2002,\textsuperscript{15} has been used successfully in treatment of antimony-resistant CL and VL.\textsuperscript{3-5,17-19} The most common side effects induced by this treatment include nausea, vomiting, and diarrhea. Some more rarely occurring but severe side effects are elevated hepatic transaminase, skin allergy, and renal insufficiency.\textsuperscript{10}
MIL is the first oral treatment option for *Leishmania* infection.\(^4,5,16,20\) While oral administration makes treatment with MIL highly attractive, our lack of knowledge regarding the specific MOA in leishmaniasis infection and the emergence of resistance is of concern. In the absence of directly observed therapy (DOT), widespread misuse of this self-administered drug could contribute to the rapid emergence of MIL resistance in the field. Further, clinical failures and relapses have been observed in phase IV clinical trials in India and Nepal.\(^16\) As MIL was originally developed as a cancer treatment, our specific knowledge of the mechanism of MIL-resistance is limited to defects in drug internalization (inward translocation of MIL) and increased drug efflux.\(^18\) Early studies detail specific point mutations in the miltefosine transporter (MT) and its β-subunit, Ros3, responsible for intracellular transport of MIL.\(^18,19,21\) These specific mutations prevent translocation of MIL, suggesting a role for the MT/Ros3 complex in the development of resistance to MIL in *Leishmania* species.\(^18,19,21\)

1.6 Research Aims

My research addresses the development of drug resistance to MIL treatment of leishmaniasis. As the only oral therapeutic option for this disease, it is imperative that MOR be understood in greater detail to better identify potential drug targets for improved treatments. Initial studies performed with *in vitro* generated MIL-resistant *L. major* FVI populations indicate that resistance may not be limited to mutations in the MT or Ros3.\(^22\) Several alternative modes of action have been suggested, including effects on parasite mitochondria, lipid metabolism, and ultimately apoptosis-like cell
death. Through generation of MIL-resistant *L. major* my dissertation aims to characterize resistant populations and identify key players in the development of resistance to this vital antileishmanial agent.
CHAPTER 2:

FITNESS AND PHENOTYPIC CHARACTERIZATION OF MILTEFOSINE-RESISTANT

LEISHMANIA MAJOR

This chapter appears in part in the published manuscript “Fitness and Phenotypic Characterization of Miltefosine-Resistant Leishmania major” by Kimbra G. Turner, Paola Vacchina, Maricela Robles-Murguia, Mariha Wadsworth, Mary Ann McDowell, and Miguel A. Morales with minimal changes.

2.1 Abstract

Trypanosomatid parasites of the genus Leishmania are the causative agents of leishmaniasis, a neglected tropical disease with several clinical manifestations. 

Leishmania major is the causative agent of cutaneous leishmaniasis (CL), which is largely characterized by ulcerative lesions appearing on the skin. Current treatments of leishmaniasis include pentavalent antimonials and amphotericin B, however, the toxic side effects of these drugs and difficulty with distribution makes these options less than ideal. Miltefosine (MIL) is the first oral treatment available for leishmaniasis. Originally developed for cancer chemotherapy, the mechanism of action of MIL in Leishmania spp. is largely unknown. While treatment with MIL has proven effective, higher tolerance to the drug has been observed, and resistance is easily developed in an in vitro
Utilizing stepwise selection we generated MIL-resistant cultures of *L. major* and characterized the fitness of MIL-resistant *L. major*. Resistant parasites proliferate at a rate comparable to the wild-type (WT) and exhibit similar apoptotic responses. As expected, MIL-resistant parasites demonstrate decreased susceptibility to MIL, which reduces after the drug is withdrawn from culture. Our data demonstrate metacyclogenesis is elevated in MIL-resistant *L. major*, albeit these parasites display attenuated *in vitro* and *in vivo* virulence and standard survival rates in the natural sandfly vector, indicating that development of experimental resistance to miltefosine does not lead to an increased competitive fitness in *L. major*.

### 2.2 Introduction

#### 2.2.1 Disease Background

*Leishmaniasis* is caused by protozoan parasites of the genus *Leishmania*, and presents as a variety of clinical manifestations ranging from lesions on the skin to disseminated visceral infections.\(^7\) Cutaneous leishmaniasis (CL) often results in self-resolving lesions, whereas visceral leishmaniasis (VL) is habitually fatal when left untreated. With an annual incidence of 2 million cases and a prevalence of more than 12 million, leishmaniasis is responsible for 70,000 deaths annually.\(^{23,88}\) 88 countries have reported infection, resulting in 350 million individuals at risk for infection and an estimated 2.4 million disability-adjusted life years (DALYs).\(^{23}\) These statistics are grossly underestimated due to misdiagnosis and insufficient disease surveillance systems.
2.2.2 Life Cycle

*Leishmania* species have a digenetic life cycle including both extracellular promastigote and obligate intracellular amastigote forms. Extracellular flagellated promastigotes reside in the midgut of the phlebotomine sandfly vector. Following infection in the mammalian host, promastigotes are engulfed by macrophages where they differentiate into non-motile amastigotes in the phagolysosome. This differentiation is triggered by environmental cues, mainly pH and temperature.\(^{24}\)

2.2.3 Current Treatments

Current antileishmanial drugs include pentavalent antimony, amphotericin B, paromomycin, pentamidine, and miltefosine; most are toxic and expensive. To date, no successful vaccine exists, and the few antileishmanial drugs mentioned either risk becoming ineffective due to emerging resistance, or are limited in their use due to cost and parental administration.\(^{4,5}\) Miltefosine (MIL) is an alkylphosphocholine drug with demonstrated activity against various parasite species and cancer cells, as well as some pathogenic bacteria and fungi.\(^{15}\) Since its registration in 2002, miltefosine remains the only oral agent used for the treatment of all types of leishmaniasis. The U.S. Food and Drug Administration (FDA) recently (March 2014) approved Impavido (miltefosine) for the treatment of cutaneous, visceral, and muco-cutaneous leishmaniasis.

2.2.4 Miltefosine Resistance and Mechanism of Action in *Leishmania*

While the mechanism of action of MIL is not understood in its entirety, several studies have pointed at alterations in phospholipid metabolism, impairment of
bioenergetic metabolism, and ultimately the induction of apoptosis as potential modes of action.\textsuperscript{25-28} Knowledge of experimental MIL resistance in \textit{Leishmania} is limited to defects in drug internalization (defective inward translocation of MIL) and increased drug efflux.\textsuperscript{18} Previous investigations in \textit{L. donovani} have revealed the presence of several key point mutations in the P-type ATPase dubbed the LdMT (\textit{L. donovani} miltefosine transporter).\textsuperscript{21} However, subsequent studies demonstrated that the LdMT alone was not sufficient to facilitate translocation, leading to the identification of the β-subunit LdRos3 and its importance to the function of the LdMT.\textsuperscript{19} Mutations in the LdMT and Ros3 contribute to the MIL-resistant phenotype by significantly decreasing MIL uptake. Specifically, T420N and L856P mutations in the LdMT contributed to significantly decreased MIL uptake.\textsuperscript{21} Other mutations identified in MIL-resistant \textit{L. donovani} include W210 (LdMT) and M1 (LdRos3).\textsuperscript{29} Sequencing of the entire miltefosine transporter was performed in both \textit{L. major} and \textit{L. infantum}, and all identified sequence mutations differed from those previously detailed in \textit{L. donovani} (L856P, T420N, W210, and M1).\textsuperscript{20} In the same study, no mutations were observed in the β-subunit Ros3 in any of the MIL-resistant populations. Widespread clinical resistance has not yet been demonstrated, nonetheless two \textit{L. infantum} isolates from HIV co-infected patients have been reported to exhibit MIL resistance.\textsuperscript{30,31} The analysis of clinical isolates from patients infected with \textit{L. donovani} that had relapsed to standard MIL therapeutic regimes demonstrated that the recovered parasites were significantly more tolerant to MIL.\textsuperscript{29} None of the resistance markers i.e. point mutations aforementioned were found in the isolates. In the absence of a definitive mechanism of miltefosine resistance, the
concept of fitness or “proficiency” of drug resistant pathogens is becoming more relevant and how the acquisition of resistance may impact the life cycle of the parasite, particularly its capacity to survive both in the insect and mammalian hosts and thus its ability to compete with wild-type (sensitive) parasites.\textsuperscript{32-34} Most of these studies are focused on antimony resistance in \textit{L. donovani} and more recently, drug combinations.\textsuperscript{35} Here we present the characterization and fitness of clonal lines of \textit{L. major} that have experimentally acquired resistance to miltefosine, with relevance to survival in the mammalian host and phlebotomine vector.

2.3 Materials and Methods

2.3.1 Ethics Statement

All studies using vertebrate animals were conducted in accordance with the U. S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and followed the standards as described in the Guide for the Care and Use of Laboratory Animals. Per these standards, all vertebrate animal studies were conducted following review by the University of Notre Dame Institutional Animal Care and Use Committee under protocol #15–047 (approved October 16, 2012). The University of Notre Dame is credited through the Animal Welfare Assurance #A3093-01.

2.3.2 Cell Culture Conditions

\textit{Leishmania major} strain Friedlin VI (MHOM/JL/80/Friedlin) promastigotes were cultured at 27°C in M199 medium (medium 199 (CellGro) supplemented with 10% heat-
inactivated fetal bovine serum (FBS), 20 mM HEPES, 10 mM adenine, penicillin/streptomycin, hemin, biotin, L-glutamine, and 7.5% NaHCO₃) and passaged every 3–4 days. Macrophages (RAW264.7 cell line) were cultured at 37°C with 5% CO₂ in RPMI supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, and L-glutamine, and passaged every 2–3 days.

2.3.3 Generation of MIL-Resistant Populations

MIL-resistant cultures of L. major were generated using step-wise selection. Cultures were passaged every 3–4 days at an initial concentration of 5x10⁵ promastigotes/mL. Increasing concentrations of MIL (Sigma) were introduced to the cultures beginning with 2.5 μM MIL and successively to 5, 8, 10, 15, 20, 30, and 40 μM MIL. Cultures were exposed to an increased concentration of MIL when growth rates were equivalent to the growth rate of the wild-type (WT). To account for clonal variation, 2 clones of each resistant line were generating by plating in M199 plates as previously described 36. Clones 1 and 2 were simultaneously maintained.

Growth rates were measured for each set of resistant populations and compared with the WT strain. Parasites were counted at an initial concentration of 5x10⁵ parasites/mL and growth was measured daily using a Neubauer chamber until the population reached stationary phase. To further assess stability and fitness, two fluorescent FACS-based apoptotic markers were used to evaluate MIL-selection. Membrane permeability was assessed using the kit YO-PRO1 (Invitrogen) according to manufacturer’s recommendations. Briefly, samples were pelleted and washed in 1X
M199 complete media. Following the wash, samples were resuspended in 1X M199 complete media and YO-PRO (Invitrogen) and Propidium Iodide (Invitrogen) were added and incubated for 20 minutes. Exposure of phosphatidylserine (PS) residues was investigated with Annexin-V-FITC (Miltenyi Biotec) following manufacturer’s instructions. Analyses were performed in a Beckman Coulter FC500 Flow Cytometer.

2.3.4 Assessment of Drug Resistance

In order to assess the MIL-resistance achieved, the half-maximal effective concentration, EC$_{50}$, was performed using the resazurin-based CellTiter-Blue (Promega) method as previously described.$^{37}$ Cultures were counted using a Neubauer chamber. 1x10$^6$ parasites/mL were incubated for 48 hours at 27°C in M199 medium (CellGro) and appropriate concentrations of MIL (Sigma), pentamidine isethionate (Sigma), amphotericin B (Sigma), potassium antimony (III) tartrate hydrate (Sigma) and paromomycin sulfate salt (Sigma), were used in order to accurately evaluate the resistance. Solvent (DMSO) controls were used where appropriate. 100 µL from each well were incubated at 37°C at 5% CO$_2$ for 4 hours with 20 µL Cell Titer Blue (Promega). 50 µL of 10% SDS were added to each well, and fluorescence was measured (555 nm $\lambda_{exc}$/580 nm $\lambda_{em}$) using a Typhoon FLA-9500 laser scanner (GE Healthcare) and analyzed with ImageQuant TL software (GE Healthcare). EC$_{50}$ values were calculated by non-linear regression analysis using SigmaPlot (v 11.0). All experiments were done in triplicate with appropriate controls in each case.
2.3.5 Partial Sequencing of LmMT and LmRos3

Both WT and MIL-resistant cultures were sequences for previously described point mutations in the *L. donovani* MT (T421N, L856P, W210*) and Ros3 subunit (M1)\textsuperscript{29} and in *L. major* (G852D, M547del).\textsuperscript{20} DNA was amplified with primers outlined in Table 2.1. PCR product sizes ranging from 149-277 bp were purified using the GeneJET Gel Extraction Kit (Thermo) and sent to the Genomics Core Facility at the University of Notre Dame for sequencing. Sequences were analyzed using ClustalX.\textsuperscript{38}

2.3.6 RNA Extraction and Real-Time PCR Analysis

Total RNA was isolated from logarithmic and stationary phase promastigotes using Trizol Reagent (Invitrogen), reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) after deoxyribonuclease I treatment with TURBO DNA-free Kit (Ambio, Invitrogen). All qRT-PCR reactions were performed in triplicate using SYBR Green (Invitrogen) fluorescence for quantification in a 7500 Fast Real-Time PCR System (Applied Biosystems). The \( \Delta \Delta C_t \) method was used to determine relative changes in gene expression\textsuperscript{39} with data presented as fold change in the target gene expression in *L. major* MIL-resistant cultures normalized to internal control genes GAPDH and SOD, using *L. major* WT as a reference strain. Standard PCR conditions were: 95°C for 10 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. Primer design was based on nucleotide sequences of *L. infantum* genes coding for the *L. donovani* MT, *L. donovani* Ros3, SHERP, GAPDH, and SOD genes. All experiments were performed in triplicate with appropriate controls included in each case.
TABLE 2.1

PRIMER SETS USED TO TEST FOR PREVIOUSLY IDENTIFIED MUTATIONS IMPLICATED IN

*LEISHMANIA* MILTEFOSINE RESISTANCE.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
</table>
| Ld W210* | Fwd: CGA GGA AGG ACA GGC ATT TA  
  Rev: GGT CTG GCT TGC TCG TGT C |
| Ld T421N | Fwd: CTG CCT ATG ATG GAG TAC A  
  Rev: GCC TAG CCC CTT CGA CTC |
| Ld L856P | Fwd: CCA ACG ACG TGT CCA TGA T  
  Rev: AAG GTC AGC ATC CAT CCA TC |
| Ld M1* | Fwd: TAC AGC TTT TGC TGC CCT TT  
  Rev: ATA GCA GCG ACT GCC AGA AT |
| Ld SHERP | Fwd: CGA CAA GAT CCA GGA GCT GAA GGA C  
  Rev: CCT TGA TGC TCT CAA CCG TGC TG |
| Lm MT | Fwd1: CCC TTT CTG TGT TCA CAG AAA CGC GCG GG  
  Fwd2: GGA CAT CCA CCC CGG TGA CG  
  Fwd3: GGA GTA CAT GAA CAA CCG CTG GCG GC  
  Fwd4: GCC GGC AAG TCC CTG CAC AAC CGC  
  Fwd5: CGC TCT TCC GCA ACG CCA GCT GC  
  Rev: CGC TCT TCC CTC GCC AAG TGA CTA CC |
| Lm Ros3 | Fwd1: GCA CTA CTC TCA ACC TCG TGT TTG CG  
  Fwd2: CGG CCA ACG GAA CGA GTC TGG CGC C  
  Rev: GGT AAA ACT GCT CTA TTG ATG ATG GC |
2.3.7 Metacyclogenesis

Two different methods were utilized to assess metacyclogenesis as described previously.\textsuperscript{40} Briefly, a Ficoll (Sigma) gradient was set-up using 4 mL of 20\% Ficoll overlaid with 4 mL of 10\% Ficoll in M199 medium without FBS and 4 mL of 5-day stationary-phase culture in M199 medium laid on top. The step gradients were centrifuged at room temperature for 10 min at 1300 \texttimes\, g without braking or acceleration to separate out the layers. The top two layers of the gradient were recovered and the percentage of metacyclic parasites was determined by counting in a Neubauer chamber before and after the enrichment procedure. For agglutination analysis, 5-day stationary-phase cultures were pelleted and resuspended in 1 mL M199 medium (CellGro) and 10 µL peanut agglutinin (50 µg/mL) (Sigma) was added. After 30 minutes of room temperature incubation, samples were centrifuged at 200 \texttimes\, g for 10 minutes. The supernatant was recovered and the percentage of metacyclic parasites was determined by counting in a Neubauer chamber before and after the enrichment procedure. All experiments were done in triplicate.

2.3.8 Macrophage Infections

RAW264.7 murine macrophage cells were counted using Trypan Blue (Amresco) and plated at 5x10\textsuperscript{5} cells/well in 12-well plates. Infections were performed with metacyclic parasites isolated as described above. Infections were carried out at a multiplicity of infection (MOI) of 10 parasites per macrophage. Free parasites were removed by one wash with RPMI without FBS 6 h post-infection and samples were
collected a 6, 12, 24, and 48 h post-infection by DiffQuick staining of cytospin whole-cell preparations and visualized with light microscopy. All infections were done in triplicate and at least two independent experiments were performed.

2.3.9 Sandfly Infections

*Phlebotomus papatasi* (Origin: Turkey, PPTK) was reared in the Department of Biological Sciences, University of Notre Dame, according to conditions previously described. For the experiment, three-to-five day old female sandflies were used. Two groups, one experimental and one control, each containing 50 female and 10 male sandflies were placed in a 500ml plastic container (ø = 6.3 cm, height = 6.5 cm) (Thermo-Nalgene) covered with a piece of nylon mesh (0.5mm). Blood feeding was performed through a young chicken skin membrane attached to a feeding device. Prior to sandfly feeding, fresh mouse blood was heat inactivated for 30 min at 56°C. Infection of sandflies with *L. major* FVI strain promastigotes was done by addition of $1 \times 10^7$ logarithmic parasites/mL into the blood meal. Sixteen to twenty four hours after blood feeding, the presence or absence of blood in the sandfly digestive tract was verified by anesthetizing flies with CO$_2$ and observing the midgut distension under a stereomicroscope (Carl Zeiss). One week post-blood meal, midguts of blood-fed sandflies were individually dissected and thoroughly homogenized in a 30 µL PBS buffer (pH 7.4) using a hand held tissue homogenizer and pestle. Parasites were counted in a Neubauer chamber.
2.3.10 Mouse Strains and Infections

5x10^5 metacyclic parasites isolated by peanut agglutinin (see above) from stationary cultures of *L. major* FVI were injected subcutaneously in the left hind footpad of Balb/c mice, as previously described.\(^{40}\) Lesion development was monitored by measuring weekly the thickness of the footpad using a Vernier caliper. Number of parasites at lesion site were enumerated by limiting dilution assay.\(^{42}\) Cell lines were passaged at least once through mice before performing *in vivo* virulence studies to minimize the loss of virulence after prolonged in vitro culture.

2.3.11 Statistics

Significance was determined by p-values calculated from a two-tailed student’s T-test in GraphPad Prism 6.0 unless otherwise stated.

2.3.12 Accession Numbers

*L. donovani* MT: GenBank accession number AY321397.1; *L. donovani* Ros3: GenBank accession number DQ205096.1; SHERP: GenBank accession number XM_001683391; GAPDH: GenBank accession number XP_001684904, and SOD: GenBank accession number XP_001695502.

2.4 Results and Discussion

2.4.1 Selection of MIL-Resistant Populations of *L. major*

*L. major* FVI MIL-resistant parasites were generated using step-wise selection up to 40 µM MIL. Parasites were unable to proliferate in higher MIL concentrations, likely
due to reaching the critical micellar concentration of MIL leading to degradation of the membrane due to the detergent effects of MIL.\textsuperscript{43} FVI WT promastigotes were plated in solid M199 media and two random clones were used for MIL selection in flasks. In order to assess the degree of MIL-resistance in our lab populations of \textit{L. major} we measured EC\textsubscript{50} values using the resazurin-based CellTiter-Blue (Promega) assay. MIL-resistant cultures exposed to the highest concentrations of MIL (30 µM, 40 µM), and labeled R30 and R40 herein, have accordingly higher EC\textsubscript{50} values than R10 and R20 (Figure 2.1). MIL-resistant cultures growing in the absence of MIL exhibited lower EC\textsubscript{50} values than their counterparts under constant MIL-selection. However, it is important to note that this decreased EC\textsubscript{50} value of MIL-resistant \textit{L. major} is still higher than the EC\textsubscript{50} of WT \textit{L. major} cultures (Figure 2.1, dotted line) after at least 95 passages (2 passages per week, ca 11 months). This suggests that once any degree of resistance is accrued MIL-resistant cultures do not revert back to WT phenotype, despite the removal of MIL selective pressure (Figure 2.1). It is worth noting that a different resistant phenotype may be obtained if drug selection is performed in axenic promastigotes or intracellular amastigotes, as shown for paromomycin selection in antimony-resistant \textit{L. donovani}.\textsuperscript{31,44}

2.4.2 Phenotypic Characterization

We next determined any difference in growth patterns between the sensitive (WT), resistant (R30) and resistant grown in the absence of MIL (R30no) \textit{L. major} populations. Growth curves showed that MIL-resistant \textit{L. major} proliferation is similar to \textit{L. major} WT and cured lines (Figure 2.2), indicating that increased MIL exposure has
no effect on proliferation in *L. major*. We used a FACS-based approach to detect two different apoptotic markers i) membrane permeability and ii) PS exposure to determine the response of parasite to stress after MIL selection. *L. major* R30 cell lines exhibit minimal stress and are comparable to WT populations judging the histogram levels corresponding to Annexin V and YO-PRO as analyzed by flow cytometry (Figure 2.3).

Experimental MIL-resistance in *L. donovani* has previously been attributed to identified point mutations in the MT and Ros3 subunit (T421N, L856P, W210, and M1).\(^{45}\) We sequenced the regions of the transporter and subunit in two independent clones of the R40 line (highest concentration; R40.1 and R40.2) that had been under drug selection for at least 75 passages. As shown in Table 2.2, these mutations were not found in our lab populations. These results are in accordance with previous characterization of were pinpointed for this study: a three-nucleotide deletion (M547del) and a transition mutation (G852D).\(^{20}\) As seen in Table 2.2, our lab populations displayed identical sequences to WT. Although our data do not eliminate the possibility of other unidentified genetic mutations having a role in MIL-resistance in *L. major*, it is interesting to observe that even at higher concentrations (R40) and after long-term exposure to MIL (at least 75 passages) none of the reported mutations were found.

2.4.3 Cross-Resistance of MIL-resistant *L. major* Populations to Other Antileishmanials

We investigated the possibility of any conferred resistance to alternative antileishmanial treatments by measuring EC\(_{50}\) values as described in Materials and Methods. No cross-resistance was found in any of the R30 clones or cured lines to
Figure 2.1: Susceptibility of MIL-resistant *L. major* FVI populations generated by step-wise selection and determined by EC50 analysis. 

1x10⁶ Log-phase parasites were incubated in the presence of a range of drug concentrations at 27°C, and the surviving cells were quantified with Cell Titer Blue proliferation assay using a Typhoon FLA-9500 laser scanner. Populations of parasites were grown in increasing concentrations of MIL ranging from 10 µM (R10) to 40 µM (R40), showing increased resistance to MIL. Horizontal dashed line represents WT threshold for MIL resistance. “Rno” are resistant lines grown in the absence of MIL for at least 75 passages. Results are the average of triplicate experiments ± SD.
Figure 2.2. Growth curves of *L. major* WT and MIL-resistant promastigotes growing in the presence of 30 µM MIL or absence of MIL selection. Log-phase promastigotes cultures were counted daily until they reached stationary phase. Concentration was determined microscopically by counting in a Neubauer chamber. Results are the average of triplicate experiments ± SD.
Figure 2.3: Flow cytometry analysis of MIL-resistant *L. major* promastigotes. WT, *L. major* FVI promastigotes grown in 40uM MIL, and R40 promastigotes where the MIL selection has been withdrawn, using two different apoptotic markers (A) Annexin V and (B) YO-PRO.
**TABLE 2.2**


<p>| | | | |</p>
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</tr>
<tr>
<td><em>L. major</em> R40 clone 2</td>
<td>1635</td>
<td>CCATCTGAATCCA</td>
<td></td>
</tr>
</tbody>
</table>

Previously identified mutations were sequences and are indicated with an asterisk (*) and highlighted in bold font, using *L. major* FVI wild-type as the reference strain. No mutations were detected in any of the resistant lines.
amphotericin B, antimony (III) and paromomycin (Table 2.3). Interestingly, miltefosine resistance significantly increases the sensitivity of the parasite to treatment with pentamidine 3-fold lower than WT (Table 2.3). When MIL has been withdrawn, the sensitivity of the parasite to this particular treatment is restored to levels comparable with the WT (Table 2.3), suggesting a potential synergistic mechanism. A similar synergy has been reported for sitamaquine/pentamidine combinations in *L. donovani*,\(^46\) although the use of a combined therapy of miltefosine and pentamidine is hindered by the high toxicity of pentamidine.\(^47\) Lastly, treatment of R30 MIL-resistant cultures with paromomycin had a significant effect on the sensitivity (ranging from 2-4 fold lower than WT) of one of the clones (R30.2), indicative of potential clonal variability.

2.4.4 Metacyclogenesis in MIL-Resistant Parasites

*Procyclic* *L. major* promastigotes differentiate into highly virulent metacyclic promastigotes during metacyclogenesis.\(^48\) This process occurs in the midgut of sandflies and can be mimicked *in vitro* when acidification occurs in the medium. Due to the lack of phenotypic differences in our clonal lines we performed the following *in vitro* and *in vivo* experiments with the R40.2 line. We enriched metacyclic promastigotes by Ficoll 400 step gradient and peanut agglutination, as described in Materials and Methods. Analyses of metacyclogenesis showed that *L. major* R40 had higher percentages (2-fold) of metacyclics than *L. major* WT (Figure 2.4, right panel). qRT-PCR was used to amplify the SHERP gene, which is almost exclusively and highly expressed in infective and non-
## TABLE 2.3

MIL RESISTANCE IN *L. MAJOR* FVI PROMASTIGOTES DOES NOT CONFER CROSS-
RESISTANCE TO ALTERNATIVE ANTILEISHMANIALS.

<table>
<thead>
<tr>
<th>Drug</th>
<th>LmWT</th>
<th>LmR30.1</th>
<th>LmR30no.1</th>
<th>LmR30.2</th>
<th>LmR30no.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miltefosine (µM)</td>
<td>6.68±0.43</td>
<td>142.29±6.24</td>
<td>24.08±0.17</td>
<td>107.80±6.16</td>
<td>21.39±0.43</td>
</tr>
<tr>
<td>Amphotericin B (nM)</td>
<td>52.36±11.37</td>
<td>69.61±15.05</td>
<td>44.38±4.03</td>
<td>46.36±0.57</td>
<td>64.13±14.41</td>
</tr>
<tr>
<td>Antimony (III) (µM)</td>
<td>11.66±0.08</td>
<td>5.43±0.70</td>
<td>10.04±0.98</td>
<td>9.43±0.99</td>
<td>14.77±2.22</td>
</tr>
<tr>
<td>Pentamidine (µg/ml)</td>
<td>3.23±0.43</td>
<td>0.93±0.10</td>
<td>3.13±0.35</td>
<td>1.71±0.12</td>
<td>3.57±0.34</td>
</tr>
<tr>
<td>Paromomycin (µM)</td>
<td>161.91±9.78</td>
<td>41.56±6.52</td>
<td>108.56±3.02</td>
<td>99.54±6.82</td>
<td>94.05±3.00</td>
</tr>
</tbody>
</table>

1x10⁶ parasites/mL were incubated in increasing concentrations of amphotericin B (nM), pentamidine (µg/mL), paromomycin (µM), or antimony (III) (µM) for 48 hours at 27°C, using solvent controls where appropriate. Surviving cells were determined through the proliferation Cell Titer Blue assay using a Typhoon FLA-9500 laser scanner. Results are the average of triplicate experiments ± SD. (-) indicates no cross-resistance demonstrated as compared to WT, and (s) indicates an increased susceptibility to treatment.
Figure 2.4: Metacyclogenesis in WT and MIL-resistant *L. major*. *L. major* promastigotes resistant to MIL exhibit increased metacyclogenesis as determined by qRT-PCR of SHERP expression relative to housekeeping gene GAPDH and normalized to WT expression levels (left). 5-day stationary parasites were subjected to peanut agglutination and Ficoll-400 gradients and percentage of metacyclics is shown (right). Results are the average of triplicate experiments ± SD. Statistical differences determined with a Student’s t test relative to control values (*p<0.05).
replicative stages of the parasite.\textsuperscript{49} SHERP expression was significantly elevated in R40 parasites (Figure 2.4, left panel), confirming our metacyclic enrichment approaches. Increased metacyclogenesis has been reported in antimony-resistant \textit{L. donovani} clinical isolates,\textsuperscript{50} and metacyclogenesis is regarded as a major contributor to the fitness of the parasite. In New World cutaneous species, \textit{L. Mexicana} resistant to Glibenclamide, an ATP-binding-cassette (ABC)-transporter blocker exhibited a reduced expression of the Meta-1 protein.\textsuperscript{51}

2.4.5 \textit{In Vitro} and \textit{In Vivo} Infection Studies

The stationary phase-specific differences of R40 primed us to study their capacity to infection RAW264.7 murine macrophage cells. We routinely passage our \textit{L. major} cell lines through Balb/c mice to compensate for the loss of virulence due to \textit{in vitro} culture. 5-day stationary cultures were subjected to peanut agglutination, and R40 and WT liens were incubated with RAW264.7 cells at a multiplicity of infection of 10 metacyclics per host cell. Intracellular parasite burden was determined by nuclear staining and microscopy at 6, 12, 24, and 48 h post infection. Initial levels of R40 infections are comparable to the control (Figure 2.5A). A significant difference in R40 infectivity was apparent 48 hours post infection by over 20\% (Figure 2.5B). Pentamidine-resistant \textit{L. mexicana} showed no differences in the \textit{in vitro} infectivity in resident mouse macrophages when compared with the wild-type clone.\textsuperscript{52}

In contrast, higher metacyclogenesis levels in clinical isolates of \textit{L. donovani} resistant to antimony translated into higher \textit{in vitro} infection levels.\textsuperscript{50}
Figure 2.5: Host cell infection assay. Early stages of macrophage invasion are similar between *L. major* WT and R40, as determined by infection of RAW264.7 murine macrophages. Metacyclic parasites were incubated in the presence of macrophages at a MOI of 10 metacyclic parasites per macrophage and cells were collected at 6h, 12h, 24h, and 48h. Samples were stained and infection was determined through light microscopy. (A) The percentage of infected macrophages, and (B) the number of parasites/100 cells were recorded. Results are the average of triplicate experiments ± SD. Statistical differences determined with a Student’s *t* test relative to control values (*p*<0.05; **p**<0.01).
We next investigated the virulence of WT and R40 using an established experimental mouse infection.\textsuperscript{53} Control and R40 were normalized for virulence through one passage in Balb/c mice.\textsuperscript{54} $10^5$ WT and R40 metacyclic parasites were inoculated into the hind footpad of groups of five-six female Balb/c mice. A Vernier caliper was used to monitor lesion formation by measuring the increase in footpad size weekly. Control parasites attained a lesion size of ca. 4mm, 5 weeks after inoculation and resulted in necrotic lesions (Figure 2.6). Interestingly, R40 were highly attenuated and lesions were only apparent 4 weeks after infection. Our observations \textit{in vitro} with R40 cells showing a decreased infectivity and intracellular proliferation seem to have extended well to an \textit{in vivo} mouse model. Amphotericin-resistant \textit{L. Mexicana} parasites were able to infect Balb/c mice, but the resulting lesion growth was slower than that after infection with susceptible parasites.\textsuperscript{55} In contrast, several clinical isolates of \textit{L. donovani} resistant to pentavalent antimonials showed a greater virulence in a mouse model of visceral leishmaniasis.\textsuperscript{56} Importantly, our data suggest that metacyclogenesis alone is not a reliable marker of fitness, at least in MIL-resistant \textit{L. major}, and \textit{in vitro} and \textit{in vivo} studies are necessary to further assess its competitive fitness. In this scenario, the \textit{L. major}/MIL combination resembles the reduction in fitness widely observed in \textit{Plasmodium falciparum} populations resistant to chloroquine.\textsuperscript{57}

2.4.6 Sandfly Infection Studies

Fitness of \textit{Leishmania} parasites is linked to transmission success in the natural insect vector, therefore we tested whether MIL resistance would impact the capacity of
Figure 2.6: Virulence of WT and MIL-resistant *L. major*. R40 demonstrate attenuated virulence *in vivo* compared with WT promastigotes. $1 \times 10^6$ WT (n=5) and R40 (n=6) metacyclic promastigotes were injected into the footpads of female Balb/c mice. Lesion size was recorded weekly by taking measurements of footpad thickness with a Vernier caliper, results are averages ± SD.
*Leishmania* to survive in the natural sandfly vector. Three-to-five day old female *Phlebotomus papatasi* (Origin: Turkey, PPTK) sandflies were infected with $1 \times 10^7$ logarithmic parasites/mL as described in Materials and Methods. 24h post-blood meal, the presence or absence of blood in the sandfly digestive tract was verified and one-week post-blood meal, 9 midguts of blood-fed sandflies infected with WT and 14 midguts from the R40 group were individually dissected. Parasite load per individual midgut was assessed. No significant differences were observed between the two groups (Figure 2.7) suggesting that MIL resistance does not affect the survival capacity of *L. major* in the natural vector.

In summary, as shown for *L. donovani*, the generation of experimental resistance to MIL is easily achieved by step-wise selection in *L. major*. Axenic resistant promastigotes proliferate as control cells, and the phenotype is stable. As suggested by our data, metacyclogenesis is an important process in the life cycle of the parasite, but should be carefully interpreted as a fitness marker. A combination of *in vitro, in vivo,* and vector studies are necessary to fully assess the competitive fitness of MIL-resistant *L. major*, and studies would be further strengthened with the use of recent clinical isolates of both MIL-sensitive and MIL-resistant *L. major* parasites. Further studies will attempt to understand the impaired ability of MIL-resistant *L. major* to survive in the mammalian host at the molecular level. Overall, our findings are relevant for current and future antileishmanial chemotherapy strategies.
Figure 2.7: WT and R40 *L. major* parasites exhibit comparable ability to colonize and survive in the sandfly vector. *P. papatasi* were fed with heat-inactivated fresh mouse blood mixed with $1 \times 10^7$ parasites/mL of both *L. major* WT and R40. Blood-fed sandflies (WT: n=9, R40: n=14) were maintained for one week on a sucrose diet, after which the midgut was dissected. Midguts were placed in 50 µL 1X PBS and crushed with a pestle. Parasite presence in each midgut was assessed by counting with a Neubauer chamber. No significant differences were observed.
CHAPTER 3:

NEW INSIGHTS INTO MILTEFOSINE RESISTANCE IN LEISHMANIA MAJOR

This chapter was completed in collaboration with Paola Vacchina and Miguel A. Morales.

3.1 Abstract

Drug resistance is becoming an increasing problem in the treatment of leishmaniasis. This disease is caused by protozoan parasites of the Leishmania species. Here, we focus on the development of resistance to treatment with miltefosine in Leishmania major, the causative agent of cutaneous leishmaniasis. Miltefosine is the first oral therapeutic available for treatment of the leishmaniases. In light of emerging resistance to pentavalent antimonials, the preferred treatment option, miltefosine has emerged as a front-runner due to the ease of administration in disease endemic areas. However, clinical trials have revealed the presence of miltefosine-resistant populations of Leishmania, and drug resistant parasites are easily generated in vitro. Originally developed as a cancer chemotherapeutic, the mechanism by which this antileishmanial agent acts on the parasite is not well understood. Following a gel-based proteomics...
analysis of wild-type versus miltefosine-resistant *L. major*, several proteins were identified as potential key players in this drug-parasite interaction. Previous studies have implicated an apoptotic-like programmed cell death as a result of miltefosine exposure. Here we build on the role of the parasite stress response to further investigate this interplay between miltefosine and *L. major*.

3.2 Introduction

3.2.1 Disease Background

Cutaneous leishmaniasis (CL) is one of the clinical manifestations caused by trypanosomatid parasites of the *Leishmania* species. The disease is largely characterized by the formation of ulcerative lesions on the skin of the mammalian host. These lesions are self-healing in the absence of treatment, but can leave behind disfiguring scars, contributing to the roughly 2.4 million disability-adjusted life years attributed to this disease. Worldwide, the leishmaniases are responsible for an annual incidence of 2 million cases and a total prevalence of 12 million, leading to approximately 70,000 deaths annually. It is important to note that these numbers are likely underestimations of the actual global impact, due to the severe underreporting of disease incidence. This is likely due to the similarity of disease symptoms with other common ailments, mainly in the absence of the characteristic lesions. Current treatments are limited, none of which having been expressly developed for use against leishmaniasis. These treatments have severe side effects and require daily administration from a medical professional for approximately one month. In some of
the more endemic areas, resistance has emerged against the preferred first-line

treatment, pentavalent antimonials.\textsuperscript{3,5,15,17} With a treatment failure rate of roughly 65%,
treatment success is rapidly deteriorating.\textsuperscript{1,17}

Miltefosine (hexadecylphosphocholine, MIL), introduced as an antileishmanial

agent in 2002,\textsuperscript{15} demonstrated success against antimony-resistant leishmaniasis.\textsuperscript{3-5,17-19}

As the first oral treatment option for \textit{Leishmania} infection,\textsuperscript{4,5,16,20} MIL is highly attractive.

However, our lack of knowledge regarding the specific MOA in leishmaniasis infection

and the emergence of resistance is of concern. In the absence of directly observed
therapy (DOT), the ease of MIL treatment administration could contribute to rapid

emergence of MIL resistance in the field. Despite the relatively short duration of MIL as

an alternative treatment, resistance has already been observed in clinical trials in India

and Nepal.\textsuperscript{16}

3.2.2 Intracellular Regulation of Miltefosine

   Early investigations into the development of miltefosine (MIL)-resistance in

\textit{Leishmania} revealed deficiencies in the translocation of MIL across the cell membrane.\textsuperscript{21}

Further investigation into this revealed the presence of several key point mutations in

the P-type ATPase dubbed the LdMT (\textit{L. donovani} miltefosine transporter).\textsuperscript{21} However,

subsequent studies demonstrated that the LdMT alone was not sufficient to facilitate

translocation, leading to the identification of the $\beta$-subunit LdRos3 and its importance to

the function of the LdMT.\textsuperscript{19} Perez-Victoria \textit{et al.}\textsuperscript{19} found that expression of both LdMT

and LdRos3 was required for phospholipid translocation across the membrane—
absence of LdRos3 resulted in a phenotype similar to that observed in LdMT -/- parasites, further supporting the role of LdRos3 in phospholipid transport across the membrane.\textsuperscript{19} Further, mutations in the LdMT and Ros3 contribute to the MIL-resistant phenotype by significantly decreasing MIL uptake.\textsuperscript{21,45} Specifically, T420N and L856P mutations in the LdMT contributed to significantly decreased MIL uptake.\textsuperscript{21} Other mutations identified in the MIL-resistant \textit{L. donovani} include W210 (LdMT) and M1 (LdRos3).\textsuperscript{29} Sequencing of the entire miltefosine transporter was performed in both \textit{L. major} and \textit{L. infantum}, and all identified sequence mutations differed from those previously detailed in \textit{L. donovani} (L856P, T420N, W210, and M1).\textsuperscript{20} In that same study, no mutations were observed in the β-subunit Ros3 in any of the MIL-resistant populations.\textsuperscript{20} In the absence of point mutations in the MT/Ros3 implicated in MIL-resistance, we cannot rule out that other membrane transporters play key roles in the active translocation of MIL.

3.2.3 Regulation of Gene Expression in \textit{Leishmania}

Proteomics analyses are vital to our understanding of gene expression and regulation in \textit{Leishmania} species. The \textit{Leishmania} genome is polycistronic, with multiple genes encoded by a single promoter. Thus, protein coding genes are organized into long gene clusters,\textsuperscript{59} and the resulting polycistronic RNAs are post-transcriptionally processed into mature mRNAs by concomitant trans-splicing and polyadenylation. Additionally, trypanosomatids appear to have few of the regulatory transcription factors,\textsuperscript{60} and transcription initiates at fewer than 200 sites in the entire genome.\textsuperscript{61}
Therefore, due to the lack of regulatory transcription factors, differences in expression cannot be determined at the transcript level.\textsuperscript{60,61} Accordingly, proteomics analyses are vital to our understanding of gene expression and regulation in \textit{Leishmania} species, facilitating determination of post-transcriptional differences in expression.

3.2.4 \textit{Leishmania} Stress Response and Programmed Cell Death

\textit{Leishmania} parasites experience drastic changes in their environment during transmission events between the sandfly vector and a mammalian host. In order to be able to adapt to these changing environmental conditions, they increase expression of stress proteins such as heat-shock proteins (HSPs).\textsuperscript{62} It has been shown that HSPs are differentially phosphorylated during stress.\textsuperscript{63} We speculate that members of the mitogen-activated protein kinase (MAPK) family are responsible for this post-translational modification, which may play a role in modulating the stress response in \textit{Leishmania}.\textsuperscript{64}

Release of cytochrome \textit{c} from mitochondria, a proapoptotic marker, has been documented in \textit{Leishmania}.\textsuperscript{65,66} However, the utility of cytochrome \textit{c} as an apoptotic marker is limited in \textit{Leishmania} until downstream signaling events are better understood (binding to apoptotic protein activating factor, Apaf-1, or activation of caspase homologs).\textsuperscript{65} Further, apoptosis in mammalian cells is highly regulated by caspases and members of the Bcl-2 family. Evidence for Bcl-2 protein homologs is minimal, and no conserved caspases have been found in \textit{Leishmania}.\textsuperscript{65,66} However, there has been evidence for caspase-like activity implicated in parasite programmed cell
death (PCD).\textsuperscript{65,66} This is thought to be due to the presence of caspase or cathepsin homologs that can recognize the Asp-Glu-Val-Asp (DEVD) peptide, and this approach has been successfully performed in \textit{L. donovani}.\textsuperscript{65-67} Here, we use a gel-based proteomics approach to pinpoint specific players in the MIL mechanism of action. Further, we investigate specific stress response and cell death mechanisms implicated in the development of MIL resistance in \textit{L. major}.

3.3 Materials and Methods

3.3.1 Cell Culture Conditions

\textit{Leishmania major} strain Friedlin VI (MHOM/JL/80/Friedlin) promastigotes were cultured at 27°C in M199 medium (medium 199 (CellGro) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM HEPES, 10 mM adenine, penicillin/streptomycin, hemin, biotin, L-glutamine, and 7.5% NaHCO$_3$) and passaged every 3–4 days. Macrophages (RAW264.7 cell line) were cultured at 37°C with 5% CO$_2$ in RPMI supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, and L-glutamine, and passaged every 2–3 days.

3.3.2 Generation of MIL-Resistant Populations

MIL-resistant cultures of \textit{L. major} were generated as previously described.\textsuperscript{22} Briefly, cultures were passaged in increasing concentrations of MIL (Sigma) up to 40 μM MIL. Cultures were exposed to an increased concentration of MIL when growth rates were equivalent to the growth rate of the wild-type (WT).
Growth rates were measured for each set of resistant populations and compared with the WT strain. To further assess stability and fitness, two fluorescent FACS-based apoptotic markers were used to evaluate MIL-selection. Membrane permeability was assessed using the kit YO-PRO1 (Invitrogen) according to manufacturer’s recommendations. Exposure of phosphatidylserine (PS) residues was investigated with Annexin-V-FITC (Miltenyi Biotec) following manufacturer’s instructions. Analyses were performed in a Beckman Coulter FC500 Flow Cytometer.

3.3.3 Assessment of Drug Resistance

In order to assess the MIL-resistance achieved, the half-maximal effective concentration (EC$_{50}$) was performed using the resazurin-based Cell Titer-Blue (Promega) method as previously described.$^{36}$ Cultures were counted using a Neubauer chamber. 1x10$^6$ parasites/mL were incubated for 48 hours at 27°C in M199 medium (CellGro) and appropriate concentrations of MIL (Sigma) and CspA (Fluka Analytical) were used in order to accurately evaluate the resistance. Solvent (DMSO) controls were used where appropriate. 100 µL from each well were incubated at 37°C at 5% CO$_2$ for 4 hours with 20 µL Cell Titer Blue (Promega). 50 µL of 10% SDS were added to each well, and fluorescence was measured (555 nm λ.exc/580 nm λ.em) using a Typhoon FLA-9500 scanner (GE Healthcare) and analyzed with ImageQuant TL software (GE Healthcare). EC$_{50}$ values were calculated by non-linear regression analysis using SigmaPlot (v 11.0). All experiments were done in triplicate with appropriate controls in each case.
3.3.4 Two-Dimensional Gel-Based Proteomics

Protein extracts from *L. major* pLEXSY-mock, and cyp40-pLEXSY in 2X Hygromycin promastigotes were differentially labeled with the spectrally resolvable Cy3 and Cy5 as previously described. A pool of extracts was labeled with Cy2 for normalization purposes following the manufacturer’s recommendations (GE Healthcare). Following labeling, proteins were precipitated using a 2-D Clean-Up kit (GE Healthcare), allowing for quantitative precipitation and removal of interfering substances, such as detergents, salts, lipids, phenolics, and nucleic acids.

Isoelectric focusing (IEF) of 100 or 120 µg of protein was carried out using an Ettan IPGphor 3 System (GE Healthcare) at 20°C with 13cm non-linear DryStrip (pH 4-7). Strips were passively rehydrated overnight at room temperature in rehydration solution (GE Healthcare) containing 0.5% IPG buffer 4-7 and the sample. The IEF maximum current setting was 50µA/strip. The following conditions were programmed for IEF: 100V gradient step for 5h, 300V gradient step for 5h, 1000V gradient step for 2h, 6000V gradient step for 8h, and 6000V gradient step for 5h (60550 Vh). Following IEF, strips were equilibrated in two different solutions for 15min each (6M urea, 75mM Tris/HCl pH 8.8, 29.3% glycerol, 4% SDS, 0.002% bromophenol blue) supplemented with 65mM DTT and 13.5mM iodoacetamide, respectively. The strips were transferred to SDS polyacrylamide gels and sealed with 0.5% agarose in 25mM Tris-base, 0.19M glycine, 0.2% SDS, 0.01% bromophenol blue. Electrophoresis was carried out in an SE 600 Ruby cooled electrophoresis system (GE Healthcare) using 12.5% SDS-PAGE gels and two-step runs (1 W/gel for 15 min and 7 W/gel for 5 h).
After electrophoresis, gels were scanned on a Typhoon FLA 9500 Imager (GE Healthcare) using 488/520 nm for Cy2, 532/580 nm for Cy3, 633/670 nm for Cy5 and 100μm as pixel size. Gel images were normalized by adjusting PMT voltage to obtain appropriate pixel value without any saturation. Images were analyzed with Decyder v. 6.5 (GE Healthcare) and Delta2D v.4.3 software (Decodon). Gels were matched or warped and spots detected across all images. A 2-fold difference in abundance, with p-values <0.05, was considered significant for the expression profiles. Polyacrylamide gels were then fixed in 50% methanol and 7% acetic acid and stained using SYPRO Ruby total protein gel stain (Life). Spots of interest were manually excised from gels using a blue-light transilluminator (Life).

The gel spots were subjected to reduction with 55mM dithiothreitol (Sigma-Aldrich) in 25mM ammonium bicarbonate (Fisher Scientific) at 56°C for 1 hour followed by alkylation with 100mM iodoacetamide (Sigma-Aldrich) in 25mM ammonium bicarbonate at room temperature in the dark for 45 min. The spots were washed with 25mM ammonium bicarbonate for 10min followed by two consecutive washes with 25mM ammonium bicarbonate in 50/50 acetonitrile:water for 5 min each. The spots were placed in a vacuum concentrator to dry completely before the addition of 12.5ng trypsin gold (Promega) to each gel spot. The spots were kept at 4°C for 30min to swell and then were incubated at 37°C overnight. Following trypsin digestion, the supernatant was collected. Peptides were further extracted from the gel spots with two consecutive additions of 50% acetonitrile/45% water/5% formic acid to the spots followed by 30min of vortexing. The two sets of extracts were combined with the
supernatant from each gel spot and then vacuum concentrated to 10µL. Each concentrated digest was desalted with C18 Siptip (EMD Millipore) according to the manufacturer’s instructions. The desalted digests were then dried down in a vacuum concentrator and reconstituted in 10µL of 0.1% TFA in water. A 2µL aliquot of each gel digest was injected onto a nanoAcquity UPLC (Waters Corporation) with a BEH300 C18 100µMx100mm column (Waters Corporation) with 1.7µm particle size. A gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was performed starting with 2% B held for 6 min and then ramping to 40% B for 40min and 90% B for 43min. The column was washed with 90% B for 7min and then re-equilibrated with 98% A: 2% B. The nanoAcquity was coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Corporation) for data dependent scans of the digested samples in which the top nine abundant ions in a scan were selected for CID fragmentation. The UPLC-MS/MS chromatograms and spectra were analyzed using Xcalibur software (Thermo), and the extracted data were searched against the L. major custom database via Mascot and/or Protein Pilot. Search criteria included a global modification of carbamidomethylation on the cysteines. Proteins identified had less than a 1% false discovery rate.

3.3.5 Gene Cloning and Transfection

The coding sequence for the LmCyp40 gene (GenBank accession number: LmjF35.4770) was amplified from 1 ng genomics DNA of L. major Friedlin-VI WT strain using the primer pair Fw LmCyp40 (5’ - ACCAGATCTATGCCGAACACACACTGC- 3’) and Rv
LmCyp40 (5’ - CGCTCTAGACGAGAACATCTTCTTGAG - 3’). The reaction was performed in a final volume of 25 µL using Taq polymerase (Life) following the manufacturer’s recommendation. The product (1,065 bp) was cloned into pGEM-T (Promega) to create the construction pGEM-T-LmCyp40 and subsequently fully sequenced. LmCyp40 fragment was excised from the p-GEM-T construct using BglII (New England Biolabs) and XbaI (New England Biolabs), and subsequently ligated into pLEXSY-hyg (Jena Bioscience GmbH) to create the construction pLEXSY-LmCyp40. An episomal transfectant was established by electroporation of 4x10⁷ L. major WT promastigotes with 75 µg of recombinant vector. Transfected cells were selected in media containing 2X hygromycin (Corning). Parasites transfected with the empty vector, pLEXSY-hyg, were used as mock-controls. Additionally, pLEXSY-LmCyp40 construct was linearized using Swal (New England Biolabs) and gel-purified using the Freeze N’ Squeeze kit (Bio-Rad) following the manufacturer’s recommendation. 6 µg of linearized vector was transfected by electroporation into 4x10⁷ L. major WT promastigotes.

3.3.6 Western Blot

Briefly, cells were washed once by centrifugation with PBS and lysed in 500 µL lysis buffer supplemented with a cocktail of protease inhibitors (Complete Mini tablets, Roche Applied Science). Proteins were separated in 4%-12% Bis-Tris NuPAGE gels (Invitrogen) and electroblotted onto PVDF membranes (Pierce). Proteins were revealed using the following antibodies: Cyp40, rabbit polyclonal secondary antibody (Pierce, IL), mouse monoclonal anti-a-tubulin antibody (Sigma), anti-mouse HRP conjugated
secondary antibodies (Pierce, IL). After washing, blots were developed using SuperSignal chemiluminescent detection system (Pierce) and visualized on X-ray film.

3.3.7 Immunofluorescence

Log-phase cultures were pelleted, washed twice with ice-cold PBS and deposited onto slides with a Shandon Cytospin3 (Thermo Scientific) at 800 RPM for 5 minutes. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton-X 100 in PBS. Cells were washed with 100 mM glycine in PBS three times and blocked with 10% goat serum (Invitrogen) in IF buffer (130 mM NaCl, 7 mM Na₂HPO₄, 3.5 mM NaH₂PO₄, 7.7 mM NaH₃, 0.1% BSA (Millipore), 1.2% Triton-X 100, 0.5% Tween-20). Slides were stained with Cyp40 diluted 1:200 in IF buffer. For secondary visualization, slides were treated with AlexaFluor® 488 (Invitrogen A11034) at 1:200 in IF block buffer. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI)(Invitrogen) and mounted with ProLong® Gold Antifade Reagent. Images were collected on an Applied Precision DeltaVision OMX.

3.3.8 RNA Extraction and Real-Time PCR Analysis

Total RNA was isolated from logarithmic and stationary phase promastigotes using Trizol Reagent (Invitrogen), reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) after deoxyribonuclease I treatment with TURBO DNA-free Kit (Ambio, Invitrogen). All qRT-PCR reactions were performed in triplicate using SYBR Green (Invitrogen) fluorescence for quantification in a 7500 Fast Real-Time PCR System (Applied Biosystems). The ΔΔCT method was used to determine relative changes in
gene expression with data presented as fold change in the target gene expression in *L. major* MIL-resistant cultures normalized to internal control genes GAPDH and SOD, using *L. major* WT as a reference strain. Standard PCR conditions were: 95°C for 10 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. Primer design was based on nucleotide sequences of *L. major* genes coding for the *L. major* Cyp40, SHERP, GAPDH, and SOD genes. All experiments were performed in triplicate with appropriate controls included in each case.

### 3.3.9 Metacyclogenesis

A density gradient was utilized to assess metacyclogenesis as described previously. Briefly, a Ficoll (Sigma) gradient was set-up using 4 mL of 20% Ficoll overlaid with 4 mL of 10% Ficoll in M199 medium without FBS and 4 mL of 5-day stationary-phase culture in M199 medium laid on top. The step gradients were centrifuged at room temperature for 10 min at 1300 x g without braking or acceleration to separate out the layers. The top two layers of the gradient were recovered and the percentage of metacyclic parasites was determined by counting in a Neubauer chamber before and after the enrichment procedure. All experiments were done in triplicate.

### 3.3.10 Assessment of Parasite Stress Response

Exposure of phosphatidylinerine (PS) residues was investigated with Annexin-V-FITC (Miltenyi Biotec) following manufacturer’s instructions. Analyses were performed in a Beckman Coulter FC500 Flow Cytometer.
3.3.11 Measurement of ROS Levels

Cultures were counted using a Neubauer chamber. 1x10^6 parasites/mL were incubated for 48 hours at 27°C in M199 medium (CellGro) and appropriate concentrations of MIL (Sigma) were used in order to induce a stress response. 100 µL from each well was incubated at 37°C at 5% CO₂ for 2 hours with 10 µM carboxy-H₂DCFDA (Life Technologies). The resulting reaction was read at 530 nm using an excitation wavelength of 485 nm on a SpectramaxM5 plate reader (Molecular Devices).

3.3.12 Mitochondrial Membrane Potential (Δψₘ)

Cultures of *L. major* promastigotes were grown in the presence of absence of drug (MIL) to both log and stationary-phase conditions. Cultures were pelleted and resuspended in 1X PBS containing 100nm TMRE (Molecular Probes). Samples were incubated in the dark for 30 min at room temperature. The resulting Δψₘ was measured using a BD LSRFortessa X-20 flow cytometer.

3.4 Results and Discussion

3.4.1 Determination of Proteins Potentially Implicated in Resistance

Populations of *L. major* Friedlin-VI (FVI) resistant to MIL were generated and characterized as previously described. Protein extracts were prepared from *L. major* FVI WT and populations resistant to 10µM MIL. Lysates were separated two-dimensionally by pl and size using IEF with subsequent SDS-PAGE, revealing several differences between WT and *L. major* populations resistant to 10µM MIL. Three proteins
were determined of interest to this particular study as down-regulated in MIL-resistant populations (Figure 3.1, Table 3.1), including cyclophilin-40 (Cyp40), heat shock protein 70 (HSP70), and heat shock protein 83 (HSP83, analogous to heat shock protein 90 in mammalian systems). All three proteins are of particular interest to this study, having previously been identified in stage-specific phosphorylation.\textsuperscript{63} Cyp40 is an immunophilin characterized by its chaperone activity as a peptidyl-prolyl isomerase (PPIase), and is known to complex with HSP-90.\textsuperscript{68-70} Yau \textit{et al.}\textsuperscript{69} showed that Cyp40 has been tied to defects in the parasite during stationary-phase growth, further increasing interest in this particular protein.\textsuperscript{69} Cyp40 down-regulation in MIL-resistant populations was validated by western blot (Figure 3.2), expression of which was restored upon withdrawal of MIL selective pressure from culture conditions (Figure 3.2).

3.4.2 Generation and Stability of Overexpression System

We adopted a gain-of-function approach to investigate the potential role of cyclophilin-40 (cyp40) in miltefosine-resistance in \textit{L. major}. The \textit{L. major} cyp40 sequence was transfected into populations of \textit{L. major} FVI wild-type (WT) parasites with the plasmid pLEXSY-hyg2 (Figure 3.3A). Successful overexpression of cyp40 in \textit{L. major} FVI (Cyp40-pLEXSY) was achieved in comparison to FVI WT mock (pLEXSY-mock) (Figure 3.3B). Overexpression of cyp40 was further confirmed by challenging populations of FVI pLEXSY-mock and FVI Cyp40-pLEXSY with cyclosporin A (CspA), an inhibitor of cyp40.\textsuperscript{69,70} A greater concentration of CspA was required to have an effect on Cyp40-pLEXSY populations that was required for pLEXSY-mock populations, indicating increased cyp40
Figure 3.1: Proteomics analysis of *L. major* WT and MIL-resistant populations. Total protein was extracted from *L. major* resistant to 10µM MIL (R10, orange) and wild-type (WT, blue). Lysates were separated two-dimensionally by pI and size, stained with Sypro Ruby, and imaged on a Typhoon FLA 9500 Imager. Gel images were overlaid to determine differences in protein expression levels between the two populations. After mass-spectrometry analysis of several excised spots, three proteins were identified (heat shock protein 70, heat shock protein 83, and cyclophilin-40) at lower levels in the R10, to be of further interest.
TABLE 3.1

MASS SPECTROMETRY PROTEIN IDENTIFICATION

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Protein samples were excised from a 2D SDS-PAGE gel. Spots were prepared for mass spectrometry analysis and analyzed with mascot identification.
Figure 3.2: Expression of Cyclophilin-40 (Cyp40) in *L. major*. Total protein extracts were prepared from *L. major* wild-type (WT), parasites resistant to 30µM MIL in continued drug selection (R30+), and parasites resistant to 30µM MIL in which the drug pressure has been withdrawn (R30-). Lysates were immunoblotted for Cyp40, using α-tubulin as a loading control. Cyp40 appears to be expressed at lower levels in R30+ compared to WT expression levels. When MIL selection is removed (R30-), Cyp40 expression is restored to WT-levels.
expression levels (Figure 3.3C).

After successful generation of the Cyp40-pLEXSY overexpressing line, we proceeded to characterize the population as compared to pLEXSY-mock so as to determine any differences in parasite behavior. Cyp40-pLEXSY parasites demonstrated comparable growth to the pLEXSY-mock populations in terms of both proliferation and density at stationary phase (Figure 3.4A). Challenge with MIL revealed that Cyp40-pLEXSY populations were more susceptible to MIL (Figure 3.4B). This change prompted further investigation into known defects of the MIL-resistant parasites. Based on previous findings, MIL-resistant populations have a higher rate of metacyclogenesis than WT *L. major.*\(^{22}\) MIL-resistant parasites demonstrated an increase in metacyclogenesis, and we validated that Cyp40 was expressed at lower levels in these MIL-resistant populations. Accordingly, it was hypothesized that with an increase in MIL-susceptibility there would be a decrease in metacyclogenesis—a trend that we observed (Figure 3.4C). Until recently, this finding would have indicated that Cyp40-pLEXSY parasites demonstrate a decrease in overall fitness, further indicating an unfavorable overexpression. However, we previously demonstrated that metacyclogenesis alone is not an accurate indicator of parasite fitness in *Leishmania spp.*\(^{22}\)

In an effort to further assess the differences in Cyp40-pLEXSY, we performed 2D-DIGE to investigate potential interaction partners or other effects of Cyp40 overexpression in *L. major*. Following mass spectrometry analysis of several excised spots (Figure 3.5), we identified protein disulfide isomerase (PDI) as being expressed at higher levels in Cyp40-pLEXSY as compared to pLEXSY-mock. This is in accordance with
Figure 3.3: Generation and validation of episomal construct for overexpression of Cyp40 in *L. major*. (A) We used the pLEXSY plasmid with the selective marker hygromycin to transfect Cyp40 into *L. major* FVI wild-type parasites. After selection, we (B) immunoblotted Cyp40-pLEXSY and pLEXSY-mock parasites with Cyp40 to validate the overexpression. Cyp40-pLEXSY lysates display a higher amount of Cyp40 as compared to pLEXSY-mock. Overexpression was further validated by assessing (C) EC$_{50}$ values when parasites were challenged with Cyclosporin a (CspA). A higher concentration of CspA is required to have an effect on Cyp40-pLEXSY parasites. ** *p < 0.001*
Figure 3.4: Characterization of Cyp40-pLEXSY episomal overexpression. (A) Cultures were counted daily for one week using a Neubauer Chamber. Cyp40-pLEXSY parasites demonstrate comparable growth to pLEXSY-mock parasites. (B) Cultures were started at 1x10^6 parasites/ml and incubated with several different MIL concentrations for 48 hours. Cyp40-pLEXSY parasites are more susceptible to MIL (12.44±0.67, p=0.17) than pLEXSY-mock parasites (16.33±1.56). (C) Metacyclogenesis was assessed with a Ficoll gradient as described in Materials and Methods. Observed trends indicate Cyp40-pLEXSY displays lower levels of metacyclogenesis (3.40±0.91, p=0.058) than pLEXSY-mock (5.59±1.12). Results are the average of triplicate experiments ± SD. Statistical differences determined with a Student’s t test relative to control values (*p<0.05).
our previous findings, as PDI is known to partner with PPIases (such as Cyp40) to increase efficiency of protein folding. These PDI-PPI complexes are also known to associate with HSP90 during client protein maturation. Unfortunately, we were unable to continue this particular line of investigation. Levels of Cyp40 expression in Cyp40-pLEXSY decreased to pLEXSY-mock Cyp40 levels. We suspect that this loss is a result of suspected toxicity due to the unfavorable nature of this particular protein overexpression, as far as susceptibility to MIL and decreased metacyclogenesis.

Following the loss of our episomal overexpression system, we opted for a linear integration approach to further investigate the role of cyp40 in the development of MIL resistance in L. major. Using the pLEXSY-hyg2 plasmid, the L. major cyp40 sequence was transfected into FVI WT populations of L. major and we successfully confirmed increased expression levels of Cyp40 by immunoblot (Figure 3.6A). Expression levels were further confirmed with qPCR (Figure 3.6B) and immunofluorescence (Figure 3.6C).

In order to characterize our new Cyp40 overexpressing line (Cyp40integ), we performed another growth curve. The Cyp40integ parasites proliferate comparable to WT during logarithmic and early stationary phase growth, but experienced difficulty surviving in late stationary phase growth conditions (Figure 3.7). Interestingly, between WT and Cyp40-/- populations there were morphological differences observed during stationary phase, in which Cyp40 -/- populations exhibited a more spherical shape as opposed to the more characteristic spindle shape of WT L. major. This shape is often connected with cell stress and apoptotic death, and was observed in Cyp40integ parasites during stationary phase conditions (data not shown).
Figure 3.5: 2D-DIGE between Cyp40-pLEXSY and pLEXSY-mock. After labeling with Cy-dyes, total protein extract was separated two-dimensionally by pI and size and imaged on a Typhoon FLA 9500. Resulting images were overlaid to determine differences in protein expression levels between the two populations. To visualize and excise spots, gels were stained with Sypro Ruby, and imaged on a Typhoon FLA 9500 Imager. After mass-spectrometry analysis of several excised spots, one was determined to be a protein of interest. Cyp40-pLEXSY (orange) have higher levels of PDI as compared to pLEXSY-mock (blue).
Considering the link between late stationary phase and metacyclogenesis in *Leishmania*, we evaluated the ability of Cyp40integ parasites to differentiate into metacyclic promastigotes. Similar to Cyp40-pLEXSY, Cyp40integ demonstrates a significantly lower rate of metacyclogenesis than the WT populations, as determined by both qRT-PCR of the SHERP gene, and a ficoll density gradient (Figure 3.8). This decrease in metacyclogenesis, coupled with the decreased ability of Cyp40integ to survive stationary phase growth conditions, lead us to investigate potential deficiencies in the parasite ability to survive stressful conditions.

3.4.3 Cyp40 Role in *L. major* Stress Response and Programmed Cell Death

Observed morphological changes in Cyp40integ stationary phase parasites (different from WT) point to defects in the parasite ability to survive stressful conditions. Further, the decreased ability of Cyp40integ parasites to survive in stationary phase conditions coupled with lower rates of metacyclogenesis implicate the involvement of the parasite stress response and potential interactions in the apoptotic cascade in *Leishmania*. In order to determine the ability of Cyp40integ to respond stressful conditions, we used flow cytometry to look at the apoptotic marker Annexin V. WT parasites provided a baseline stress level, while Cyp40integ parasites had a small population experience stress during normal growth conditions (Figure 3.9A). This indicates that Cyp40integ populations are constantly experiencing higher levels of baseline stress than WT populations, further substantiating the hypothesis that overexpression of Cyp40 is unfavorable.
Figure 3.6: Validation of linear integration for overexpression of Cyp40 in *L. major*. (A) *L. major* wild-type (WT) and Cyp40 overexpressing (Cyp40integ) were deposited by cytospin, fixed and stained for Cyp40 (green). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). Images were collected on an Applied Precision DeltaVision OMX. (B) Total protein extracts of WT and Cyp40integ were immunoblotted with Cyp40 to validate the overexpression. Cyp40integ lysates display a higher amount of Cyp40 as compared to WT, confirmed by additional densitometry analysis. (C) Cyp40integ promastigotes exhibit heightened levels of Cyp40 as determined by qRT-PCR of Cyp40 expression relative to housekeeping genes (GAPDH, SOD) and normalized to WT expression levels. Results are the average of triplicate experiments ± SD. Statistical differences determined with a Student’s *t* test relative to control values (*p*<0.05).
Figure 3.7: Growth curve of *L. major* FVI WT and Cyp40integ. Cultures of WT and Cyp40integ were counted daily using a Neubauer chamber. Populations demonstrate comparable growth during logarithmic phase. Cyp40integ displays difficulty surviving late stationary phase conditions. Results are the average of triplicate experiments ± SD.
Figure 3.8: Cyp40integ displays decreased metacyclogenesis as compared to WT. Results were determined by qRT-PCR of SHERP expression relative to housekeeping gene GAPDH and normalized to WT expression levels (left). 5-day stationary parasites were subjected to Ficoll-400 gradients and percentage of metacyclics is shown (right). Results are the average of triplicate experiments ± SD. Statistical differences determined with a Student’s t test relative to control values (*p<0.05).
Continuing the investigation of Cyp40 in the regulation of the stress response and potentially PCD, we investigated reactive oxygen species (ROS) in our populations. Due to the role of ROS in the regulation of cell death, we hypothesized that Cyp40integ would have higher levels of ROS than WT parasites. Cyp40integ exhibited higher basal levels of ROS as compared to WT (p<0.05, Figure 3.9B). Accordingly, we wanted to determine if Cyp40integ would respond to stressors more severely than WT parasites. It has been demonstrated that in Leishmania, MIL exposure increases ROS production in drug-sensitive parasites.\(^72\) After a 48h incubation with 8µM MIL, ROS levels were measured and it was determined that Cyp40integ parasites had significantly lower ROS than WT parasites (Figure 3.9B). This indicates the potential for an increased ability of Cyp40 overexpressing parasites to combat stressful conditions, likely due to the heightened basal levels of stress Cyp40integ experiences.

With increasing ROS generally comes a decrease in mitochondrial membrane potential (\(\Delta\psi_m\)), another classical characteristic in apoptosis.\(^72\) Since trypanosomatids each only have a single mitochondria, maintaining \(\Delta\psi_m\) is crucial for cell survival.\(^73,74\) Given the lower ROS levels of Cyp40integ, we hypothesized that we would observe a similar phenomenon when we looked at \(\Delta\psi_m\). Using the mitochondrial dye tetramethylrhodamine ethyl ester (TMRE), we were able to measure the depolarization of the mitochondrial membrane by flow cytometry. After overnight incubation with 20µM MIL, Cyp40integ exhibited a lower \(\Delta\psi_m\) as compared to WT populations (Figure 3.9C), while no \(\Delta\psi_m\) was observed in populations of L. major resistant to 30µM MIL (data not shown). With WT parasites representing basal levels of the parasite response
Figure 3.9: Cyp40integ stress response as compared to WT. A) Annexin V staining of WT (green) and Cyp40integ (red), p=0.02. Cyp40integ has a higher resting level of stress as compared to WT. B) Basal ROS levels of WT and Cyp40integ (p=0.04), and ROS levels after 48h incubation with 8uM MIL (p=0.02). Cyp40integ displays a higher base level of ROS, with fewer ROS when challenged with MIL as compared to WT ROS levels. C) (left) Basal mitochondrial membrane potential of WT (pink) and Cyp40integ (blue). (right) mitochondrial membrane potential after overnight incubation with 20uM MIL between WT (pink) and Cyp40integ (blue). WT demonstrates a higher level of mitochondrial membrane depolarization compared with Cyp40integ. All experiments were performed in triplicate ± SD. *p<0.05
to PCD, we see modulations in the parasite ability to respond to stressors. This suggests that MIL-resistant parasites are able to suppress their individual stress response so as to evade drug-induced cell death. While initially the overexpression of Cyp40 seems unfavorable, it does seem to merit some consideration in the ability of the parasite to decrease the magnitude of the stress response.

Together, these data implicate the involvement of Cyp40 in the regulation of drug-induced cell death in *Leishmania major*. Cyp40 appears to be a highly regulated protein in *Leishmania* spp. Yau *et al.* demonstrated stationary-phase defects in their Cyp40 -/- *L. donovani* parasites. Our findings support the opposing effect, where there are both drawbacks and benefits to the presence of increased levels of Cyp40, further establishing Cyp40 as a highly regulated protein in *Leishmania*. Cyp40 is known to complex with HSPs, but to date little has been revealed about the role of this protein in trypanosomatid parasites. Specifically, Cyp40 has been linked to stage-specific functions in morphogenesis, motility, and metacyclogenesis. Further, Cyp40 -/- parasites were unable to establish intracellular infection *in vivo*, lending to the possibility of further defects in the ability of the parasite to undergo metacyclogenesis and survive under stressful conditions. Here, we introduce a potential model for the mechanism of MIL in *L. major*, with Cyp40 as a protein potentially implicated in the regulation of an apoptotic-like mechanism of PCD.

Cyp40 falls under the divergent loop family of proteins, with a PPIase domain (drug binding domain) and TPR domains (bind HSP90). Furthermore, Cyp40 acts as part of a signaling mechanism in response to oxidative stress through the formation of
disulfide bonds in an oxidizing environment.\textsuperscript{75} Therefore, the idea that PDI was upregulated in Cyp40 overexpressing parasites is intuitive. PDI is responsible for protein folding and the introduction and correction of disulfide bonds, and is known to play a role in bacterial virulence.\textsuperscript{76} Previously, PDI has been implicated in parasite pathogenicity and identified as a virulence factor in \textit{Leishmania}. Additionally, Achour \textit{et al.} showed that inhibition of PDI with zinc bacitracin resulted in attenuated virulence in \textit{Leishmania}.\textsuperscript{76} Due to the high level of conservation across \textit{Leishmania} species and presence in both promastigotes and amastigotes, PDI has been identified as a possible drug target in \textit{L. major}.\textsuperscript{77}

The structure of MIL consists of a positively charged quarternary amine group, a negatively charged phosphoryl group, and a fatty acid tail\textsuperscript{15} presenting an ideal site for oxidation. We posit that MIL functions to induce oxidative stress in \textit{Leishmania}, triggering depolarization of the mitochondrial membrane through generation of ROS, ultimately leading to cell death (Figure 3.10). In MIL-resistant \textit{L. major}, the parasite is potentially able to suppress the expression of Cyp40, thereby shutting down the stress response and drug-induced oxidative stress (Figure 3.10).

Cyp40 is a highly regulated protein where either too much or too little of the protein can have seemingly detrimental results for the parasite during stationary phase or metacyclogenesis. While the exact mechanism of action of MIL in \textit{Leishmania} remains largely unknown, many of the other drugs currently used for the treatment of leishmaniasis have been associated with an apoptotic-like mechanism of cell death, including Sb(III), amphotericin B, and pentamidine.\textsuperscript{72} Further investigation into the role
Figure 3.10: Proposed model for the role of Cyp40 in stress response against drug (MIL) pressure in *L. major*.
of this protein in *Leishmania* PCD is necessary to more fully understand the mechanism at work here, both in terms of the parasite stress response and the development of resistance to MIL and other antileishmanial agents.
CHAPTER 4:

SO LONG AND THANKS FOR ALL THE FISH

Work from the first half of my doctoral career, this chapter appears in part in the published manuscript “Whirling disease dynamics: An analysis of intervention strategies” by Kimbra G. Turner, Matthew J. Smith, and Benjamin J. Ridenhour with minimal changes.

4.1 Abstract

Whirling disease (WD), a severe and widespread disease of salmonids, is caused by the myxosporean parasite *Myxobolus cerebralis*. It is further characterized by a unique two-host life cycle, utilizing the oligochaete *Tubifex tubifex* as an intermediate host. *M. cerebralis* is an invasive species that has been affecting populations in the United States including epidemics that killed in excess of 90% of populations in Colorado and Montana streams within the past 20 years. Currently, there is no known cure for WD, and the accepted method of control is removal of infected fish from the population. We have created a compartmental model of the WD system in order to assess more efficient means of control and management of the disease. Using data
gathered from the literature, we used Bayesian model fitting to estimate model parameters and estimated that $R_0 \approx 1.51$ (95% CI: 1.39, 1.72), a value which implies that WD can be controlled using available strategies. To this end, we posit several implies that WD can be controlled using available strategies. To this end, we posit several parameters that we expect to be most influential to WD propagation, namely: release of triactinomyxons by *T. tubifex*, release of spores by salmonids, and infectious particle loads in each respective host. Based on currently available control strategies, approaches targeting the infectious particles and the oligochaete host appear the most effective alternative strategies for management and control of WD.

4.2 Introduction

Whirling Disease (WD) is a severe and widespread disease of salmonids. The causative agent of WD is the myxosporean parasite *Myxobolus cerebralis* which has a unique two-host life cycle, utilizing the oligochaete *Tubifex tubifex* as an intermediate host. WD was first observed in salmonid populations during the 1800s in Europe, with first observations in North America in the 1900s resulting from imports. *M. cerebralis* is an invasive species that has been affecting North American populations for nearly a century—it has resulted in the devastation of numerous fish populations in the United States including epidemics that killed in excess of 90% of populations in Colorado and Montana streams within the past 20 years. Currently in the US, WD can be found in at least 25 different states, though population-level effects appear to be restricted to the inter-mountain western region of the country. The spread of WD across the US
appears to be primarily mediated through the movement of infected fish or fish products by humans or predators.\textsuperscript{84} For example, WD is quite easily transferred through anthropogenic movement during recreational activities such as fishing, as anglers do not always clean their gear.\textsuperscript{82} Beyond human influences, environmental factors, density, species, and age distributions of fish populations all potentially influence the severity and spread of the disease once it is introduced.\textsuperscript{85,86}

WD is a recognized ecological concern that also exerts profound economic effects. Within private and public fish culture operations, devastating losses of trout due to WD has resulted in large economic costs; of 34.3 million trout intended for sale in 2007, 86\% were lost to various diseases, equivalent to a monetary loss of $35-60 million.\textsuperscript{81} Although this value is not specific to WD, it demonstrates the burden of disease in aquaculture as an area of interest. There is a wide range of WD severity in salmonid species, however the disease pathology of \textit{M. cerebralis} is most severe—and best studied—in the rainbow trout \textit{Oncorhynchus mykiss}. Severity in rainbow trout is still largely dependent on many other factors such as size, age at exposure, strain of trout infected, and the degree of exposure to infectious agents.\textsuperscript{87}

The high mortality rate of whirling disease has potentially devastating effects in wild populations. While biomagnification (when concentrations of pathogens increase with increasing trophic levels) has not been observed, transmission and progression of the disease depletes long-term availability of salmonids as a food source for predators and humans alike.\textsuperscript{81} There is no known cure for WD and the accepted method of control in the wild is removal of as many infected fish as possible. Doing so is complicated by
migration of fish and changing environmental conditions and is rarely effective.\textsuperscript{84,88}

While controlling WD in wild populations is of the greatest concern, controlling the damages of this disease to man-made hatcheries is just as important. In the event of an outbreak in such facilities current response policy dictates culling the resident population, disinfecting the facility, and occasionally even making structural changes to prevent future outbreaks. The consequences of imposing a culling intervention are extreme, especially when considering the ease with which epidemics arise and spread. All of this can cost hundreds of thousands of dollars and sometimes results in even more costly closures of facilities.\textsuperscript{81,84}

Because of the detrimental nature of culling populations, additional control approaches have been researched. These alternative approaches have been aimed at both physical and chemical targets in the WD system.\textsuperscript{89} Most of these alternative control strategies are aimed at control within hatcheries. Some of these treatments include chlorination, UV treatment, medication of fish with Fumagillin, alteration of pH, heat-killing, redesign of facilities, and manipulation of salinity levels.\textsuperscript{83,85,89-94} Fewer interventions have been proposed for and applied in wild populations. Field based interventions that have shown promise are the use of sand filtration and the alteration of land use (e.g. reduction in grading of livestock) to diminish sedimentation in streams.\textsuperscript{83,89,95} Regardless of whether these interventions are meant for hatcheries or for field use, little is known about what is actually required to control an outbreak of \textit{M. cerebralis}. 
Modeling disease systems can lend insight into improved methods of controlling disease outbreaks and no comprehensive model of WD dynamics has yet been published. However, some models have been published related to whirling disease. These models include Schisler and Bergersen,\textsuperscript{96} who modeled the likelihood of \textit{M. cerebralis} infection in Colorado, and Bartholomew \textit{et al.},\textsuperscript{84} who developed and performed risk analysis on several conceptual models of the introduction and spread of WD. These models however do not provide important insight into the epidemiology and prevention of this financially damaging disease.

To address the gap in our understanding of the growth, spread, and control of WD, we built an epidemiological model of this disease system using compartments representing different host and parasite states. More specifically, we present a model for microparasites with indirect life cycles that simplifies the system into susceptible and infected classes represented by a set of ordinary differential equations (ODEs). We then fit our system of ODEs to data available in the whirling disease literature, thereby calibrating the model to recent advances in the understanding of the life cycle and ecology of \textit{M. cerebralis}. Through utilization of our fitted compartmental model, we were able to account for population dynamics in the determination of effective treatment and control strategies for whirling disease.
4.3 Methods

4.3.1 Model Structure and Assumptions

We developed a compartmental model to understand how to control WD in rainbow trout using the population dynamics of *T. tubifex*, rainbow trout, and *M. cerebralis* infectious particles. As a basis for our model, we utilized the four principal factors introduced by Anderson and May\(^9^7\) and May and Anderson\(^9^8\) that drive microparasitic infections in animal populations. Specifically, the four principal factors of patterns of disease behavior are: (1) host habitat for the parasite, (2) degree of host mortality by the parasite, (3) extent of acquired immunity in the host, and (4) necessity of host transmission. We chose this modeling strategy because it closely matches the biology of whirling disease.

The life cycle of *M. cerebralis* is broken up into two stages identifiable by its two hosts: the oligochaete worm *T. tubifex* and a salmonid fish (Figure 4.1). The salmonid hosts display a myriad of symptoms that vary by species and age. Common signs of infection in fry include a blackening of the tail, erratic swimming behavior, skeletal deformations, and eventually death.\(^8^6\) To this end, we use an age structured population for our salmonids consisting of fry, juveniles, and adults. We use the term “juvenile” to indicate a fish that is no longer susceptible to death by WD but that is not yet reproductively mature. The complete listing of classes used in our compartment model is given in Table 2.2 and a flow diagram of the model is presented in Figure 4.2. Equations presented here in verbal format are formally defined in the online Appendix.
Figure 4.1: Whirling disease life cycle diagram. The cycle begins with an (1) asymptomatic salmonid, at any life stage, (2) after contact with a (8) triactinomyxon (TAM), (3) the salmonid becomes infected, (4) upon death of the infected salmonid, (5) *Myxobolus cerebralis* spores are released. The spores float throughout the water column until (6) ingested by the oligochaete *Tubifex tubifex*, the spores (7) transform into (8) TAMs, propagating the infection cycle.
### TABLE 4.1

**COMPARTMENTAL MODEL CLASS DESIGNATION FOR WHIRLING DISEASE IN RAINBOW TROUT.**

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<tr>
<td>$M(t)$</td>
<td>Number of spores at time $t$</td>
</tr>
<tr>
<td>$T(t)$</td>
<td>Number of triactinomyxons (TAMs) at time $t$</td>
</tr>
<tr>
<td>$W(t)$</td>
<td>Healthy worms at time $t$</td>
</tr>
<tr>
<td>$Y(t)$</td>
<td>Infected worms at time $t$</td>
</tr>
</tbody>
</table>
Figure 4.2: State diagram of the whirling disease epidemic model. Each box in the diagram represents a particular type of individual in our mode: dashed borders indicate salmonid host; dotted borders indicate *T. tubifex*; and solid borders indicate *M. cerebralis* infectious particles. The parameter(s) that affects the rate of movement from one class to a subsequent class is next to the arrow running between those same classes.
The life cycle of the salmonid host begins as a susceptible fry (born at rate $r_f$) which may become infected by contact with one or more infectious triactinomyxons (TAMs) at a rate of $\beta$. Since fry are more susceptible and have increased mortality, they are assigned a separate mortality rate $\gamma$. In order to fully account for the heightened mortality rates observed in fry, it is further assumed that there is no natural death of fry and infected fry do not survive to the juvenile life stage. Uninfected fry develop into juvenile salmonids (the age group representative of the time between deadly infections in fry and the more resilient adult stage) at rate $\sigma$. Therefore, we represent the dynamics of the fry in the whirling disease life cycle as:

$$\frac{d}{dt} (\text{Uninfected fry}) = \text{birth rate } r_f - \text{maturation rate } \sigma - \text{TAM infection rate } \beta$$

Equation 4.1

$$\frac{d}{dt} (\text{Infected fry}) = \text{TAM infection rate } \beta - \text{death rate } \gamma$$

Equation 4.2

Each rate in these equations is multiplied by the number of individuals in the affected life stage (e.g. birth rate x number of breeding adults); however, for terms where the number of newly infected fish is determined, the product of the number of individuals and the number of infectious TAMs is used (e.g. $\beta$ x number of TAMs x number of fry). The birth rate in the population is regulated by a carrying capacity $k_F$. 
After uninfected fry mature to uninfected juvenile salmonids, they are still at risk of infection.\textsuperscript{89} While fry are generally at an increased risk of infection, for simplicity we have assumed that all life stages (fry, juveniles, and adults) are infected at an equal rate $\beta$. We also assume that all juveniles survive to adulthood; juveniles mature to adults at a rate $\epsilon$. The juvenile life stage is thus represented as:

\[ \frac{d}{dt}(\text{Uninfected juveniles}) = \text{fry maturation rate} \sigma - \text{TAM infection rate} \beta - \text{juvenile maturation rate} \epsilon \]

Equation 4.3

\[ \frac{d}{dt}(\text{Infected juveniles}) = \text{TAM infection rate} \beta - \text{juvenile maturation rate} \epsilon \]

Equation 4.4

Once adulthood is achieved, few signs of infection are observed and many adults are considered asymptomatic. This is due to skeletal ossification, which reduced the amount and structural significance of the available cartilage.\textsuperscript{85} In accordance with our assumptions, adult salmonids become infected at the same rate $\beta$. Infected adult salmonids are dependent on the number of infected juveniles that mature and newly infected adult salmonids. All adults, infected, or uninfected, die at a mortality rate $\mu_s$. Altogether, this gives the following equations for the dynamics of adult salmonids in this system:
As the parasite develops within fish, it produces the cell types necessary to form the Myxospore, which is eventually released through death or predation of the infected fish at which time it can be ingested by *T. tubifex*. The number of free-floating spores in the water column is dependent on the death of infected fish (fry and adults) and the number of spores each infected fish carries. Therefore,

\[
\frac{d}{dt} (\text{Myxobolus spores}) = \text{fry mortality rate } \gamma 
\times \text{fry spore load } l_G + \text{adult mortality rate } \mu_S 
\times \text{adult spore load } l_A \times \text{spore uptake rate by worms } \lambda_M \times \alpha.
\]

Equation 4.7

describes the dynamics of the number of infectious myxospores in the system.

Myxospores are ingested by *T. tubifex* at a rate \( \lambda_M \times \alpha \), where they attach to the interior lining of the host worm’s intestines. The probability of *T. tubifex* becoming
infected by consumption of spores is represented by $\alpha$ and $\lambda_M$ is the number of myxospores consumed per infection event; this implicitly assumes that uptake of any number of infectious particles results in *M. cerebralis* infection. Infected *T. tubifex* suffer from reduced fecundity and feeding activity, though neither mortality nor growth are affected by infection with *M. cerebralis*.\textsuperscript{99} Oligochaetes are removed from the system through mortality at rate $\mu_W$. The dynamics of the worms are thus given by

$$\frac{d}{dt}(\text{Uninfected worms}) = \text{birth rate } r_W - \text{myxospore infection rate } \alpha - \text{mortality rate } \mu_W$$

Equation 4.8

$$\frac{d}{dt}(\text{Infected worms}) = \text{myxospore infection rate } \alpha - \text{mortality rate } \mu_W,$$

Equation 4.9

and, as is the case for the salmonid population dynamics, the birth rate of the worms is regulated by a carrying capacity, $k_W$.

Once inside the oligochaete, spores undergo reproduction. The resulting gametes fuse to form the zygotes that mature into the TAM stage, which is then excreted from the worm into the water column.\textsuperscript{85,100} The worms undergo periodic release of TAMs through defecation at a rate dependent on environmental and individual specific factors.\textsuperscript{84,100} TAMs then drift through the water column until they come into contact with a fish host. Therefore, the number of free-floating TAMs in the
water column is dependent on the oligochaete TAM load \( (l_y) \) in conjunction with TAMs per infection uptake/attachment \( (\lambda_T) \), the salmonid infection probability \( (\beta) \) and the total number of salmonids (both susceptible and infected) in the system. Thus, the final equation governing TAM population dynamics is

\[
\frac{d}{dt} \text{TAMs} = \text{TAM release rate} \times l_y - \text{TAM uptake rate} \times \lambda_T \times \beta
\]

Equation 4.10

Upon uptake, a TAM pierces the epidermis and migrates through the skin of the fish and releases germ cells with the help of surface villi and released proteases.\(^{85,100}\) Released germ cells then disperse and undergo mitosis, spreading to deeper layers of the skin and migrating into nervous tissue. The parasite then moves along the nervous system, making its way through cartilaginous tissue, which it digests for nutrients needed for replication,\(^{100}\) thus completing its life cycle. Biologically, salmonid contact with an infectious TAM is not guaranteed to develop WD;\(^{81}\) however, for the purposes of this model, it is assumed that uptake of an infectious particle results in infection with WD. This assumption is made in light of the wide range of values found in the literature (see \( l_A \) and \( l_C \) values, Table 4.2). Taken together, Equation 4.1 - Equation 4.10 represent the complex life cycle of *Myxobolus cerebralis* and the resulting number of infections in a salmonid population.
## Table 4.2

**Parameter Value Guidelines for Whirling Disease Based on Available Literature**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Variable</th>
<th>Targeted Value</th>
<th>Source(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma$</td>
<td>Maturation rate (fry $\rightarrow$ juvenile)</td>
<td>2 mos. Until fry are no longer susceptible to mortality by WD</td>
<td>Sipher and Bergersen (2005)</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Maturation rate (juvenile $\rightarrow$ adult)</td>
<td>Sexual maturity reached at 2 yrs of age</td>
<td>Sipher and Bergersen (2005)</td>
</tr>
<tr>
<td>$I_A, I_G$</td>
<td>Adult and fry myxospore load</td>
<td>0-1,000,000+ fish$^{-1}$</td>
<td>Thompson and Nehring (2000)</td>
</tr>
<tr>
<td>$I_Y$</td>
<td>$T. tubifex$ TAM load</td>
<td>$1.5 \times 10^3$ TAMs released x 4 releases per yr</td>
<td>Gilbert and Granath (2001)</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Infected fry mortality rate</td>
<td>Infected fry survive 4 mos</td>
<td>DuBey et al. (2007), Gilbert and Granath (2001)</td>
</tr>
<tr>
<td>$\mu_S$</td>
<td>Adult mortality rate</td>
<td>Total salmonid life span of 5 yrs</td>
<td>Hardy (2002)</td>
</tr>
<tr>
<td>$\mu_M$</td>
<td>Spore viability</td>
<td>3-30 yrs</td>
<td>Gilbert and Granath (2001)</td>
</tr>
<tr>
<td>$\mu_T$</td>
<td>TAM viability</td>
<td>6-15 days</td>
<td>El-Matbouli et al. (2001)</td>
</tr>
<tr>
<td>$\mu_W$</td>
<td>Worm mortality rate</td>
<td>Lifespan of $T. tubifex$ is 3 yrs</td>
<td>Gilbert and Granath (2001)</td>
</tr>
<tr>
<td>-</td>
<td>WD prevalence</td>
<td>0-100%</td>
<td>Sipher and Bergersen (2005)</td>
</tr>
<tr>
<td>-</td>
<td>Oligochaete infection prevalence</td>
<td>0.6-2.6%</td>
<td>Zendt and Bergersen (2000), Allen and Bergersen (2002)</td>
</tr>
</tbody>
</table>
4.3.2 Basic Reproductive Number of WD

The basic reproductive number $R_0$ of a disease is important because it determines the critical threshold at which a disease will “burn-out” of a population and not result in an epidemic.\textsuperscript{101} If $R_0 < 1$, the expectation is that there would not be a WD epidemic in either a fishery or a watershed. We found the analytical solution of $R_0$ for our WD model using the next generation method.\textsuperscript{101} In this method, $H_i(x)$ is the rate at which infections appear in class $i$, and $I_i(x)$ is the rate of removal/recovery of individuals from the same class.\textsuperscript{101} To generate the basic reproductive number $R_0$, the matrix-inverse $H^{-1}$ was calculated. $H$ and $I$ are matrices of partial derivatives where the $i$, $j$th entry is the partial derivative of $H_i(x)$ or $I_i(x)$ with respect to class $j$ evaluated at the disease-free equilibrium.\textsuperscript{101} The spectral ratio (largest eigenvalue) of $H^{-1}$ is $R_0$.\textsuperscript{101-103}

4.3.3 Model Fitting and $R_0$ Estimation

In order to parameterize our model we used Bayesian inference based upon values published in the literature (Table 4.2). Specifically, we used values related to model parameters to specify the means of prior distributions for each parameter. We utilized Gamma distributions for all model parameters with the exception of the infection probabilities ($\alpha$, $\beta$) for which we utilized Beta distributions. For those parameters where no information was available (such as $\lambda_M$ and $\lambda_T$), we used uninformative priors (e.g. $\lambda_M \sim \Gamma(10^{-2}, 10^6)$). A complete list of priors is given in Table A1 in the online Appendix. The data used to calculate the probability of any particular set of model parameters, $p(\text{model}|\text{data})$, was the observed frequency of infection in
worms and in rainbow trout. Values for prevalence of WD in trout come from Sipher and Bergersen,\textsuperscript{104} and values of prevalence of \textit{M. cerebralis} in \textit{T. tubifex} come from Zendt and Bergersen,\textsuperscript{105} and Allen and Bergersen;\textsuperscript{106} using these underlying data resulted in the basic probability model $p(\text{model}|\text{data}) = p(x_1^\text{c} | x_2 \sim \text{Beta}(11.5, 2.875))$

where $x_1$ and $x_2$ are the infected fraction of \textit{T. tubifex} and \textit{O. mykiss}, respectively, of the tested model.

We used a Markov chain-Monte Carlo sampling strategy with a Metropolis-Hastings proposal acceptance algorithm.\textsuperscript{107} We ran the sampler for $10^6$ generations and collected a new sample every 50 generations. The first 25\% of our samples were excluded from the posterior distribution as a burn-in period to remove dependence on the initial starting point of the sampler. In order to come up with an appropriate proposal distribution (a proposal being proposed set a parameter for our model), we utilized an adaptive proposal algorithm.\textsuperscript{108} This algorithm consisted of two stages: in the initial 1500 generations, the algorithm used a “greedy” start beginning on generation 80 where the proposal density was updated every 40 generations based only on accepted proposals from the previous 60 generations. In the second stage beginning at generation 2000, the proposal density was updated every 1000 generations based on all states of the Markov chain in the previous 1500 generations. The second stage concluded at generation 15000, at which point the proposal distribution became fixed for the remainder of the process. At the end of sampling, we examined the trace plots and posterior distributions of the 16 estimated parameters to verify that our sampler had converged on a solution.
Figure 4.3: Whirling disease dynamics in the fitted model. The two plots show how the prevalence of infection changes over time in both the salmonid (A) and oligochaete hosts (B). The dynamics shown here are based upon the parameters fit using our Bayesian MCMC approach. The targeted mean prevalence for both species are shown by the horizontal dashed lines. In the plot for the salmonid host: the solid line indicates all salmonids; the dashed line indicates adult salmonids; and the dotted line indicates sub-adults (i.e. fry and juveniles).
4.4 Results

4.4.1 Disease Equilibrium

Disease equilibria are important for a system because they represent the long-term values which the system will obtain during sustained outbreak periods (Figure 4.3). We found that only one stable equilibrium existed in this system that involved coexistence of salmonids with *M. cerebralis*. This endemic disease equilibrium was solved analytically and equilibrium equations are presented below using a hat notation. We begin with the results for healthy fry, but from thereon present results in terms of other classes of individuals for simplicity. Fully parameterized versions of these equations can be found in the online Appendix. Solving the system of ordinary differential equations (ODEs) yielded

\[
\hat{F} = \left( N^* - \frac{kF \mu s \tau_F}{T_F G} \right) \left( \frac{f^*}{y + \tau_F f^*} \right)
\]

Equation 4.11

as the equilibrium value of healthy fry during a WD outbreak, where \(N^*\) and \(f^*\) are the disease-free equilibrium values for the salmonid population size and proportion of fry, respectively. \(\hat{F}\) is the force of infection and is the product of probability of infection \(\beta\) and the equilibrium number of TAMs; the equilibrium number of TAMs is not presented here, but a Mathematica notebook containing all solutions is available upon request. In Equation 4.11, the equilibrium number of healthy fry consists of two terms, the first term is the population size of the salmonids during the outbreak and the second term is
proportion of population that belongs to the $F$-class at equilibrium. More interesting to the impact of WD on the fish population is the equilibrium number of infected fry, which is simply

$$\hat{G} = \frac{\hat{F}_T}{\gamma}.$$  

Equation 4.12

Equation 4.12 stands to reason because infected fry result from uninfected fry coming into contact with a TAM (an event dependent on the force of infection) and because fry tend to experience more severe mortality, they have their own respective mortality rate, $\gamma$. An important assumption that leads to Equation 4.12 is that infected fry can only be removed from the system through their own mortality (i.e. infected fry never mature). In summary, the numerator of Equation 4.12 is representative of entering the $G$ class, whereas the denominator is representative of exiting the class.

Uninfected juveniles ($J$) can be defined in terms of uninfected fry, such that

$$J = \frac{\hat{F}_\sigma}{\hat{F}_T + \epsilon}.$$  

Equation 4.13

Fry mature to uninfected juveniles (at rate $\sigma$), and in accordance with our model assumptions juveniles do not suffer mortality. As such, uninfected juveniles only exit the class through maturation (at rate $\epsilon$) or through infection (represented by $\mathcal{F}_I$). The
equation for infected juveniles \( \hat{R} \) is very similar to that of infected fry (Equation 4.12) and is given by

\[
\hat{R} = \frac{J}{\epsilon}.
\]

Equation 4.14

Clearly the equilibrium number of infected juveniles is dependent on the total number of healthy juveniles and the force of infection of TAMs. Since infected juveniles can only mature to infected adults (again in accordance with our model assumptions), they are removed from the juvenile class only through maturation.

The equilibrium values for the adult salmonids are probably the most important, particularly the infected adult class. Infected adult salmonids effectively act as reservoirs for \textit{M. cerebralis} until their death, at which time myxospores are released back into the system. The equation for the equilibrium value of healthy adults \( \hat{S} \) is

\[
\hat{S} = \frac{J \epsilon}{J \epsilon + \mu S}.
\]

Equation 4.15

Equation 4.15 follows the pattern observed in the previous equations: new uninfected adults are produced by the maturation of uninfected juveniles, and, likewise, uninfected adults can leave the class either by becoming infected or through natural mortality \( \mu_s \). The final equilibrium equation for our fish classes describes the number of infected adults \( \hat{A} \) as
\[ \hat{A} = \frac{\epsilon R + \mathcal{F}_2 \hat{S}}{\mu_S}. \]

Equation 4.16

This particular equation follows the same pattern with a slight difference, uninfected adults (\(\hat{S}\)) enter the infected adult class through one of two mechanisms: either infection or through maturation of infected juveniles (at rate \(\epsilon\)). As with the other classes, the denominator represents departures from the class, which in this case occurs through mortality (\(\mu_S\)).

At this point, we return to Equation 4.11, the initial representation of uninfected fry in a disease equilibrium state; using our other equilibrium state definitions (and in particular Equation 4.15 and Equation 4.16 we find

\[ \hat{F}_2 = \frac{(\hat{A} + \hat{S})\mu_S}{\sigma} \]

Equation 4.17

which is a much simplified version of Equation 4.11, defined in terms of adult salmonids (both infected and uninfected). Newborn healthy fry are entirely dependent on the replacement of adults that die at rate \(\mu_S\) in the system, as seen in the numerator. The denominator of Equation 4.17 is the loss of healthy fry due to maturation to the juveniles stage.
4.4.2 Basic Reproductive Number of WD

By applying the next-generation method, we found that the basic reproduction number for the given WD dynamics model is

\[
R_0 = 4\sqrt{\frac{l_Y\alpha O^*\beta N^*(l_c f^* + l_d (1 - f^*))}{\mu_W(\mu_M + \alpha \lambda_M O^*)(\mu_T + \beta \lambda_T N^*)}},
\]

Equation 4.18

Where \(O^*\) is the disease-free equilibrium values of the oligochaete population size.

From Equation 4.18, it is clear that when the numerator of the fraction is greater than the denominator a WD epidemic is expected (i.e. \(R_0 > 1\)). In order to better understand this \(R_0\) equation, it can be reorganized into four separate terms:

\[
R_0 = 4\left(\frac{l_Y}{\mu_W}\left(\frac{\alpha O^*}{\mu_M + \alpha \lambda_M O^*}\times\frac{\beta N^*}{\mu_T + \beta \lambda_T N^*}\times(\frac{l_c f^*}{\lambda_M O^*})\times(\frac{l_d (1 - f^*)}{\lambda_T N^*})\right)\right).
\]

Equation 4.19

Understanding the terms in Equation 4.19 provides insight into effective methods of control for WD. The first term, \(l_Y/\mu_W\), represents the release of TAMs from infected worms scaled by the death rate of the worms. This term implies that either increased worm mortality or decreased TAM loads in infected worms reduce the severity of WD outbreaks. The second and third terms, \(\alpha O^*/(\mu_M + \alpha \lambda_M O^*)\) and \(\beta N^*/(\mu_T + \beta \lambda_T N^*)\), represent the percentage of infectious particles that lead to infection (as compared to death via either \(\mu_M\) or \(\mu_T\)). However, for both of these terms, as the
product of the disease-free equilibrium population sizes and the infection parameter
grows then each term rapidly approaches the reciprocal of the uptake rate (i.e.
\[ \lim_{a_{0^*} \to \infty} = \frac{1}{\lambda_M} \text{ and } \lim_{b_{N^*} \to \infty} = \frac{1}{\lambda_T} \text{ for the second and third terms respectively}. \] In
general, we expect that disease-free equilibrium population sizes are large enough that
these two terms are well approximated by the reciprocal of their respective uptake rate.
The final term, \((f^* l_G + (1-f^*) l_A)\), represents spores released from fry \((f^* x l_G)\) and non-fry
\(((1-f^*) x l_A)\) salmonids.

The arrangement/simplification in Equation 4.19 allows for evaluation of the \(R_0\)
equation by treating each of the four terms as an individual variable such that

\[
R_0 = \frac{4}{\sqrt{w \times x \times y \times z}}
\]

Equation 4.20

where TAM release is represented as variable \(w\), \(1/\lambda_M\) as \(x\), \(1/\lambda_T\) as \(y\), and spore release
by fish as \(z\). Notably, \(w\) and \(z\) seem like likely targets for the control and prevention of
WD; these two terms consist of \(l_V\), \(l_G\), and \(l_A\) which are all load terms for different hosts,
the death rate of worms \((\mu_W)\), and the relative amount of fry in the fish population \((f^*)\).
The other two terms \((x, y)\) seem less likely targets for intervention unless the uptake
rate of TAMs by salmonids or myxospores by oligochaetes can be altered in some way.

To fully determine the validity of the model and to make more accurate
predictions regarding the utility of various intervention strategies, value ranges were
collected for each variable from available literature (Table 4.2). This allowed us to use
appropriate prior values for our Bayesian probability model so as to best imitate the
previously observed natural dynamics of the disease system. We estimate that $R_0 \approx 1.51$ (95% CI: 1.39, 1.72) (Figure 4.4). A basic reproductive number of 1.5 suggests that WD can potentially be controlled using appropriate interventions, which we discuss later. Our MCMC sampler showed good evidence of convergence for all 16 of the parameters being estimated; posterior means and confidence intervals can be found in Appendix Table A1.

Using the simplified values of Equation 4.20, it is clear to see that $R_0$ increases if $w, x, y$, or $z$ increases. Conversely, if the value of these variables is decreased by 100% (e.g. $w \rightarrow 0$), then $R_0$ goes to zero. However, all we require is for $R_0$ to be less than 1 to prevent WD outbreaks—this can be achieved by reducing a single variable by approximately 80% (Figure 4.5). The effects of multiple control strategies can also be readily assessed using Equation 4.20 (Figure 4.5). For example, to obtain $R_0 < 1$ when controlling two variables such as TAM release $w$ and spore release $z$, we predict that each variable only needs to be decreased by approximately 55% to prevent the spread of WD within the population.

4.5 Discussion

Our model allows for further assessment of potentially effective intervention methods in combating WD outbreaks. From our analytical results, based on Equation 4.19 and Equation 4.20 we predict that the variables that provide the most influence over the disease system are TAM load ($l_T$) in infected worms, spore load in fry ($l_G$), spore
Figure 4.4: Posterior distribution of $R_0$ for whirling disease outbreaks. The posterior distribution for $R_0$ was found using a MCMC process with a Metropolis-Hastings acceptance algorithm. The Markov chain was run for $10^6$ generation, with samples collected every 50 generations with the first 25% of all samples discarded as a burn-in period.

$\mu_{R_0} = 1.51067$

95% CI = (1.39345, 1.71854)
Figure 4.5: Predicted effects of control strategies on the prevention of whirling disease. Panel A shows the effect of using one control strategy on $R_0$. A reduction of nearly $\sim 80\%$ (vertical dashed line) in one of the four $R_0$ components (see main test) is necessary to produce a $R_0 < 1$, which indicates the disease would not produce an outbreak. Panel B plots the corresponding drop in WD prevalence with changes in a $R_0$ component. Clearly, the prevalence changes rapidly in a range of $R_0$ values from 1 to 1.3.

Panel C shows the reduction in $R_0$ components necessary to achieve control when two control strategies are used versus just one. $R_0$ values are indicated in the contours of the plot. When two intervention strategies are used, each component needs to be reduced by $\sim 55\%$ to achieve control of WD (dashed lines; dotted lines show one intervention strategy). Values shown are based upon $R_0 = 1.51$, as suggested by our model fitting process.
load in adult salmonids ($l_A$), and the mortality rate of worms ($\mu_w$). The relative importance of the adult versus fry spore load is determined by the age structure of the salmonid population, which is given by the proportion of fry $f^*$ in our model.

Manipulation of each of these variables yields the potential to create $R_0$ values above and below 1. Increasing worm mortality rates leads to a decrease in $R_0$, whiles increasing any of the infectious particle variables ($l_A, l_G,$ or $l_Y$) greatly increases the $R_0$ value. Our model predictions show that utilization of any single control approach in the management of whirling disease is not the most feasible course of action, given that at least an 80% increase/decrease in any particular parameter would be necessary to stop the spread of $M. cerebralis$ (and produce the corresponding 33% drop in our estimated $R_0$). Combining the effects of multiple control approaches proves to be a greater and more feasible approach.

4.5.1 Assessing Hatchery Interventions

Strategies such as chemical targeting approaches show promise for controlling WD due to their impact on TAM effectiveness. However, the potential for adverse effects on unintended targets is of concern, and thus such treatments are limited (in large part) to the hatchery environment. Chemical treatment of infected areas has been shown to be extremely effective, purging infected areas of the disease. Chlorine, a common chemical used for disinfection of aquatic environments, has prevented infection of rainbow trout fry, and is known to have an effect on spore and TAM efficacy as well. Through utilization of varying concentrations of chlorine, anywhere from 36%-
100% of spores can be killed. Our model shows that when the myxospore load ($l_A$) is decreased by 36%, $R_0$ is reduced by roughly 10.5%, well short of what is needed for control. Although chlorine is known to be a powerful disinfectant, it is not approved for use for discharge from hatcheries, making it a less than ideal tactic.

UV treatments, exhibiting effects on TAM viability, have potential for large-scale control in a fishery setting, but results are still widely variable. Hedrick et al. showed that static treatment of TAMs with UV resulted in no infections in salmonids exposed to the UV-killed TAMs. Depending on the UV dose used, WD infection was reduced by 31-100%. Our model shows that a 31% reduction in trout infections corresponds to a 21% reduction in $R_0$. This experiment also showed promise for the use of UV deactivation on a larger scale for WD control in fisheries. However, using UV and chlorine in combination is expected to only have additive benefits (rather synergistic benefits) because both treatments target TAM load in the system.

Other chemical tactics have involved variable alterations of the environment such as pH and salinity, aimed at reducing the viability of infectious particles. *T. tubifex* has shown tolerance to both in comparison with other aquatic oligochaetes. Physical modifications to the stream bed, water flow rate, temperature) show potential for influence over the disease system. Experiments have been performed investigating the effect of temperature on the aquatic oligochaete, and found that high temperatures (sublethal) terminated TAM production and overall infection. Our model shows that decreasing the numbers of spores (via reductions in $l_A$ and $l_G$) by 90% the $R_0$ value is decreased by approximately 44%, which would indicate this intervention is capable of
stopping a WD outbreak by itself. This, however, is the most favorable, upper bound of the effectiveness of heat-killing myxospores and should the effectiveness fall from 90% to below the 80% threshold, then other measures would be necessary to protect a salmonid population.

Another control-based approach is medication of fish using Fumagillin. As with other treatments, results with this method have proven to be variable in outcome.\textsuperscript{83,85,110} Chemotherapy with Fumagillin DCH medicated pellets at 0.1g per kg diet at 1% of body weight per day reduced infection from 100% severely infected to 10% with mild infection.\textsuperscript{85} Conversely, Staton \textit{et al.}\textsuperscript{110} showed that following FDA protocols for new animal drug approval, Fumagillin was not effective in WD control or prevention.\textsuperscript{83} Our model reconciles these seemingly contradictory results to some degree. By reducing the estimate of adult myxospore load by 90%, our model estimates that this would result in 42% drop in $R_0$. While this does exceed the 33% reduction necessary to control WD, should the effectiveness of Fumagilling be slightly lower, then $R_0 > 1$ and an outbreak would likely occur. Because the attack rate of \textit{M. cerebralis} on the host rises steeply, even relatively small $R_0$ values produce large numbers of infected fish (e.g. a $R_0$=1.1 still produces a fish population having over 31% disease prevalence). It is important to note that Fumagillin treatment is predicted to be more effective for populations where the age structure is skewed toward non-fry given that the treatment targets adults. However, treatment by Fumagillin is predicted to be nearly as effective as heat-killing, which targets spores from both adults and fry (compare 42% and 44%, respectively).
Stocking strategies can be manipulated to facilitate lower levels of infection despite infected environments. By extending fry rearing time in concrete tanks prior to transfer to infected earthen habitats, infection can be reduced. The concrete tank is ideal for a disease-free environment due to the lack of suitable habitat for *T. tubifex*. Since fry are much more susceptible to infection, this added step greatly reduces potential for disease propagation by limiting the worm environment, thus limiting TAM presence. By creating an environment with fewer susceptible fry, the disease should be less successful in the system. However, even assuming the myxospore load of fry is zero, negligible effects on $R_0$ result. The reason behind this somewhat counterintuitive expectation lies in the disease ecology of the WD system. Adult salmonids act as asymptomatic carriers of the disease, and are effectively a reservoir host. Thus, even completely protecting fry does little to stop the spread of WD; however, raising fry in protected environments does prevent mortality of infected fry.

4.5.2 Assessing Field Interventions

Filtration has yielded significant results and can be used successfully in a field setting. For example, Nehring *et al.* performed an experiment testing the use of sand filtration for removal of TAMs. The use of this sand filtration system at a site in Colorado (Fryingpan River drainage) resulted in a significant reduction in infection among susceptible salmonids. This particular application demonstrates that filtration can be successfully applied in a field setting. One shortcoming of this approach however, is that efficiency decreases when using higher volumes. Wagner
presents that anywhere from 10 to 100% of TAMs can be removed from the water column by filtration, depending on the mesh size of the filter used as well as the total volume being filtered. TAM reduction of 10% by filtration is relatively ineffective as a control measure, only producing a 2.5% drop in $R_0$. However, filtration of small volumes of water, when possible, shows promise as a component in an integrated control strategy.

Our model predicts that increasing worm mortality rates can impede the spread of WD. Approaches targeting the environment of the oligochaete host appear to show the most potential as a field-based intervention, because outbreaks depend on TAM loads and $T. tubifex$ mortality rates. As there is only one oligochaete species found to propagate WD, it stands to reason that this stage of the life cycle would be the optimal stage at which to intervene. Several approaches have been suggested, ranging from chemical strategies to physical and competition strategies. $T. tubifex$ resides in sediment at the bottom of lakes and streams. As such, areas with higher levels of sediments are “hot spots” for WD infection. Therefore, reduction of sediment would limit the $T. tubifex$ habitat. Regulation of processes that could contribute to sediment build up in lakes and streams (logging, construction, grazing, recreation) could prove a means of control. Doubling oligochaete mortality rates produces $R_0$ values roughly 16% lower; however, the accompanying drop in WD prevalence is predicted to be over 20%. Supporting the idea that increasing oligochaete mortality is a viable control strategy, the Bellevue-Watson Fish Hatchery in Colorado implemented a multi-pond
system which significantly reduced the available habitat for *T. tubifex* and subsequently experienced greatly reduced levels of WD.

4.5.3 Assumptions and Potential Weaknesses

Several key assumptions have been made during the creation of our WD model. Most of these assumptions were made either to keep our model analytically tractable or because there was a lack of information within the literature regarding necessary parameters. While these assumptions potentially lead to weaknesses in our conclusions, we argue that most of the assumptions have relatively little qualitative effect on any of our results.

We have assessed the potential effectiveness of intervention strategies based on their associated reduction in $R_0$. This method gives little information regarding the duration, cost, and effort associated with any intervention. That being said, two facts are true regarding any disease intervention: first, the earlier an intervention is applied the more likely it is to succeed and with less effort. Second, interventions must always be applied until new infections are occurring at “sub-replacement level”; after such a point, the disease will “burn out.” Our model predictions are most accurate if the intervention is applied at—or close to—the beginning of an outbreak. Unfortunately, there is no way to theoretically predict the necessary duration of an intervention without more specific information (such as when the intervention begins), thus we cannot speculate on this issue. To determine the needed duration of an intervention
requires active surveillance to determine the appropriate stopping point (i.e. when sub-replacement level is achieved).

Our model is designed as a ‘closed system’ where individuals do not enter or leave via immigration or emigration. A closed system closely mimics hatcheries where populations of captive fish are held in isolation, and thus our work most directly applies to interventions available for the containment of *M. cerebralis* in hatcheries. This being said, our model will still apply to wild populations where two conditions are met: first there must be no significant immigration or emigration from the population and, second, the population must be well mixed. Significant immigration or emigration would alter the system dynamics via arrival or departure of infected individuals. A well mixed population guarantees the infection process will be homogeneous across space; however—even in the case of a population not being well mixed—the dynamics of the system are likely to be comparable to those predicted by our model at the entire population scale (but potentially different at the sub-population scale). Examples of wild populations that might fit these conditions are small to medium sized lakes or perhaps even particular isolated stretches of rivers within watersheds.

We assumed that infection is possible with a single infectious particle (either a TAM or a myxospore). Because our model was fit to available data on the prevalence of WD in rainbow trout and *T. tubifex*, our assumption regarding the number of necessary particles is compensated for by the parameters controlling the probability of infection (*α*, *β*). In other words, if we were to assume more infectious particles were required to produce an infection, the probabilities of infection would need to have a corresponding
increase in order to keep prevalence at the intended levels (~80% and ~2% in salmonids and oligochaetes, respectively). Thus, this assumption has no effect on our results and conclusions, but it does mean that the estimates of $\alpha$ and $\beta$ from our Bayesian estimation represent lower bounds.

Finally, we assumed that natural mortality only occurs in adult salmonids and that there is no difference in mortality of infected and uninfected adults. The latter assumption regarding mortality was made because we found no evidence in the literature that adults carrying the WD parasite suffered elevated mortality. Thus, our assumption is in keeping with empirical evidence. The assumption that natural mortality only occurs in adults is clearly not realistic; however, we could find no data on mortality in salmonids beyond their general lifespan and this assumption makes the analysis of our model more tractable. This assumption has potential impacts on the percentage of fry in the population ($f^*$) which is important for $R_0$. However, we expect this assumption to have little impact because (susceptible) fry enter into the population only when other individuals die—due to the density-dependent growth conditions we have used. Thus, $f^*$ is dependent on mortality across all ages groups. If we allowed for mortality in other age classes but still maintained a five year lifespan, the following would happen: mortality in fry and juveniles goes up while mortality in adults goes down. The net effect is that approximately the same number of susceptible fry would be entering the population per unit time and any effects on our results should be negligible.
4.6 Conclusion

Our research predicts that utilization of combination treatment strategies shows the potential for controlling the spread of whirling disease in the United States. This is the first attempt at mathematically modeling the epidemiological dynamics of *M. cerebralis* infections. We found that the basic reproductive number of whirling disease was relatively low, which indicates that this financially damaging disease may be more feasible to control than previously thought using the appropriate strategies discussed above. Furthermore, our model demonstrates that non-chemical based approaches targeting the infectious particles, such as UV or temperature treatments or filtration, and those targeting the oligochaete hosts present the most effective means of controlling this disease and reducing disease propagation. More specifically, the combination of two or more approaches is predicted to produce a control strategy that should significantly reduce or eliminate the presence of WD with minimal adverse effects on the environment.


